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THE EXTRACELLULAR NUCLEASE ACTIVITY OF MICROCOCCUS SODONENSIS

A THESIS

Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of
MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

FACULTY OF SCIENCE

by

Sheila A. Berry

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Extracellular Nuclease Activity of Micrococcus sodonensis" submitted by Sheila A. Berry, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

An extracellular nuclease produced by Micrococcus sodonensis was isolated and purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion exchange chromatography and gel filtration. An increase in specific activity of 1400 times that of the crude material was obtained.

Production of the enzyme was demonstrated to be a function of young, rapidly growing cells and not dependent upon cell autolysis. Enzyme production occurred over a pH range of 6.5 - 8.5 in a variety of synthetic and non-synthetic media, but the presence of NH_4^+ or an α -amino acid, which will function as a source of NH_4^+ , was found to be essential for production.

The purified enzyme was studied. It was shown to be heat sensitive, the depolymerase activity being completely destroyed when heated for 5 min at 55°C . Mg^{++} and Mn^{++} were found to be essential for activity with Ca^{++} having a synergistic effect. The optimum pH for activity was 8.8. Sedimentation analyses yielded an $S_{20,w}$ of 1.92S and a molecular weight of 103,000.

Studies on substrate specificity showed the enzyme to be active against both native and denatured deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and polyadenylic acid (poly A). It possesses both phosphodiesterase and phosphomonoesterase activity, the latter being specific for the 5' ribo and deoxyribo mononucleotides, and these two activities could not be separated by any of the fractionation procedures used.

The depolymerase activity on the polynucleotide substrate was similar to that of snake venom phosphodiesterase (an exonuclease) with the exception that the final digestion products were nucleosides and inorganic phosphate rather than the corresponding 5' mononucleotides.

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LIST OF ABBREVIATIONS

TCS	Trypticase soy
OD	Optical density
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
TCA	Trichloroacetic acid
ANS	Molybdcic-aminonaphtholsulfonic acid reagent
UTCA	0.25% uranyl acetate in 10% trichloroacetic acid
TRIS	Tris(hydroxymethyl)aminomethane
RNase	Ribonuclease
DNase	Deoxyribonuclease
P _i	Inorganic phosphate
UV	Ultraviolet
pA	5' adenylic acid
Ap	3' adenylic acid
pG	5' guanylic acid
Gp	3' guanylic acid
pC	5' cytidylic acid
Cp	3' cytidylic acid
pU	5' uridylic acid
Up	3' uridylic acid
pT	5' thymidylic acid
dpA	5' deoxyadenylic acid
dpG	5' deoxyguanylic acid
dpC	5' deoxycytidylic acid
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
pTp	Thymidine-3',5'-diphosphate
Poly A	Polyadenylic acid
Py	Pyrimidine
Pu	Purine
N	Nucleoside
pN	5' nucleotide
Np	3' nucleotide
pNp	3',5'-diphosphonucleoside
Tp	3' thymidylic acid

INTRODUCTION

INTRODUCTION

Nucleases are enzymes which degrade nucleates. The existence of these enzymes in various organisms and tissues has been noted for several years but only a few have been highly purified or intensively studied. Pancreatic ribonuclease (RNase) and deoxyribonuclease (DNase) were first crystallized by Kunitz (1940, 1950) from beef pancreas, and probably have been the most intensively studied of the mammalian nucleases. The thymus DNase of Maver and Greco (1949) and the pancreatic DNase of Kunitz have served as prototypes of the DNase II and DNase I enzymes in classification studies. Other mammalian sources which have been found to contain nucleases are spleen (Catcheside and Holmes, 1947; Hilmo and Heppel, 1953; Kaplan and Heppel, 1956), kidney (Cunningham and Laskowski, 1953), serum (Kurnick, 1952) and urine (Kowlessar, Altman and Hempelmann, 1954). In fact there are probably few, if any, tissues which do not contain these enzymes. A phosphodiesterase from snake venom has been studied and methods have been described for its purification, most of the work being done on rattlesnake (Crotalus adamanteus) venom (Razzell, 1963). Nucleases have been isolated from a variety of microorganisms. Streitfeld, Hoffmann and Janklow (1962) reported DNase production in 22 strains of Pseudomonas aeruginosa. Wannamaker (1958) found 3 distinct DNases in Group A Streptococci and a fourth type was isolated by Winter and Bernheimer (1964) from the same organism. Two RNases have been isolated from Bacillus subtilis (Nishimura, 1960) and 2 from Escherichia coli (Lehman, 1963). Eaves and Jeffries (1963) reported a non-specific phosphodiesterase from Serratia marcescens. Four distinct DNases have been reported in E. coli (Lehman, 1960; Lehman, Roussos and Pratt, 1962;

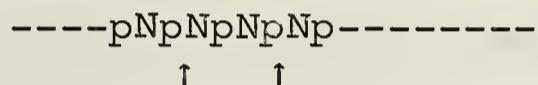
Lehman, 1963; Richardson, Sample, Schildkraut, Lehman and Kornberg, 1963) and much information has been obtained on the characterization and mode of action of these enzymes. Another microbial nuclease which is well characterized is that found in Staphylococcus aureus (Cunningham, Catlin and Privat de Garilhe, 1956) and which has been termed "Micrococcal Nuclease". A table comparing the properties of the E. coli and Micrococcal nucleases with those of the nuclease in this study, is given in the Discussion.

Most of the mammalian nucleases, with the exception of those found in urine and serum are intracellular. Of those microbial enzymes previously mentioned, all, with the exception of E. coli nucleases, are extracellular and are released into the culture medium.

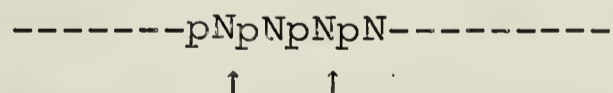
In an attempt to classify nucleases from different sources, Laskowski (1959) suggested 4 criteria; type of substrate, type of attack, products formed and preferential linkage. The division into RNases and DNases was probably the earliest division historically. It was after the crystallization of pancreatic RNase and DNase (Kunitz, 1940, 1950) that the specificity for the sugars of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) was proven and the names became established. Yet a third group of enzymes attack both RNA and DNA and the term phosphodiesterase had been applied to this group (Heppel and Rabinowitz, 1958). These workers suggested that phosphodiesterases such as snake venom may in fact be a mixture of enzymes which have not been separated by the purification procedures used. Laskowski (1961) suggests that the action of Micrococcal nuclease against both RNA and DNA may be indicative of two enzymes

incompletely separated.

The enzymes within each group vary in their mode of attack upon the substrate. Some exhibit endonuclease activity, attacking first the internal linkages in the polynucleotide chain releasing large fragments which in turn become substrates until all the sensitive bonds are hydrolysed. Micrococcal nuclease has been demonstrated to act in this manner releasing products with 3' phosphate terminated end groups (Alexander, Heppel and Hurwitz, 1961; Reddi, 1959; Sulkowski and Laskowski, 1962). The mode of attack is shown diagrammatically as:



After digestion of the DNA the products predominating were found to be Tp, Ap and the dinucleotides of A and T and it was concluded that the bond preferentially attacked was of the type XpTp and XpAp when DNA was used as a substrate (Dirksen and Dekker, 1960; Roberts, Dekker, Rushizky and Knight, 1962). Pancreatic DNase also exhibits endonucleolytic activity releasing products with 5' phosphate terminated end groups (Potter, Brown and Laskowski, 1952; Vanecko and Laskowski, 1961). The mode of attack is as shown:

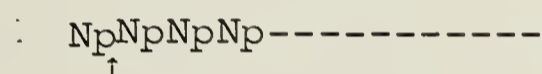


Of the dinucleotides isolated from the products the sequence d-pPy.pPu predominated heavily over the sequence d-pPu.pPy and from this it was concluded that the d-pPu.pPy bond is the one preferentially attacked. The products of the endonucleases vary from mononucleotides through di-, tri-, etc., up to octanucleotides.

Exonucleases, on the other hand, attack from one end of the polynucleotide chain releasing mononucleotides. Snake venom phosphodiesterase has been shown to attack in this manner from the 3' hydroxyl end of the chain, releasing 5' mononucleotides.



It is specific for polynucleotides with a free 3' hydroxyl group (Razell and Khorana, 1959) and has been designated phosphodiesterase I. Another type of exonuclease is spleen phosphodiesterase (phosphodiesterase II) which attacks the polynucleotide from the 5' hydroxyl terminus, releasing 3' mononucleotides.



Its action is dependent upon the presence of a free 5' hydroxyl group (Razzell and Khorana, 1961).

It has been suggested (Weckman and Catlin, 1957; Di Salvo, 1958) in the case of S. aureus, that there is a correlation between pathogenicity and DNase production with no DNase being produced by the coagulase - negative strains. Since the coagulase test is unwieldy and not always reliable these workers suggested that DNase production might be a useful means of identifying pathogens. It was observed in our laboratory that Micrococcus sodonensis, a non-pathogenic member of the Micrococcaceae, produced a very active extracellular DNase while Micrococcus violagabriellae (a reported pathogen) did not.

This study was undertaken to isolate, purify and characterize the M. sodonensis enzyme and to compare its properties

with those of other Micrococcal nucleases as well as nucleases from other sources.

PART I

PRODUCTION OF ENZYME AND DETECTION OF ACTIVITY

PART I

Materials and Methods1. Culture and Growth Conditions

The organism used in these studies was Micrococcus sodonensis, ATCC 11880. Fluid cultures were grown in flasks with vigorous aeration and all cultures were grown at an incubation temperature of 30°C. Stock cultures were maintained on Trypticase Soy (TCS) agar at 4°C.

Standard inocula were prepared from 24 hr cultures grown on TCS agar. Cells were washed in sterile distilled H₂O, resuspended to yield an optical density (OD) of 1.0 at 600 mμ and 0.1 ml of this suspension was used as an inoculum.

Cell growth was estimated both by measuring OD at 600 mμ, and by dry cell weight. Twenty four hr old cells, grown in TCS broth, were harvested, washed and dried to constant weight at 105°C and a standard curve was prepared relating dry weight to OD.

2. Media Employed(a) Synthetic Medium

The synthetic medium employed was that described by Campbell, Evans, Perry and Niven (1961a). The concentrations indicated are per 100 ml final volume.

CaCl ₂	1.0 mg
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.08 "

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.0	mg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.8	"
H_3BO_3	0.4	"
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.1	"
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.1	"
FeSO_4	0.4	"
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	20.0	"
KCl	50.0	"
Versene	0.05	"
Sodium glycerol phosphate	10.0	"
Biotin	0.001	"
Lactic Acid	500.0	"
Tris(hydroxymethyl)aminomethane (TRIS)	365.0	"
NH_4Cl (if present)	50.0	"
Glutamic acid	500	"
pH was adjusted to 7.3		

(b) TCS broth (Baltimore Biological Laboratories)

pH 7.3.

Solid media were obtained by the addition of 1.5% agar (Difco) to the preceding. Media were sterilized by autoclaving at 121°C for 15 minutes.

3. Enzyme Assay Procedures

(a) Screening Technique for DNase Production

A modification of the method used by Jeffries, Holtman and Guse (1957) was employed. Plates of TCS agar, containing 0.2% (w/v) DNA, were inoculated with a diametric streak of M. sodonensis. After 48 hr incubation the plates were flooded with cold 10% tri-chloroacetic acid (TCA) and examined for zones of clearing around the area of culture growth.

(b) Diffusion Plate Assay

The method used was that reported by Berry and Campbell (1965).

Diffusion Plate Assay Agar was prepared as follows:

	<u>per 100 ml</u>
MgCl ₂ ·6H ₂ O	0.081 gm
CaCl ₂	0.044 "
Difco Agar	1.50 "
TRIS	0.121 "

pH adjusted to 8.8

The mixture was heated to melt the agar, cooled to 50°C, then 100 mg of C grade herring sperm DNA (CalBio-chem) was added and the mixture was poured into petri dishes or onto glass slides and allowed to solidify. Fractions to

be tested for enzyme activity were applied to 6 mm Whatman #1 filter paper discs, dried and laid on the solidified assay agar or added to antibiotic assay cups embedded in the agar. After incubation at 37°C for 18 hr the following developing procedure was employed.

The plates were (i) flooded with cold 10% TCA, incubated in the cold for 15 min, the excess acid removed then (ii) flooded with molybdic-aminonaphtholsulfonic acid (ANS) reagent, a modification of the Fiske-Subbarow reagent for inorganic phosphate determinations.

The ANS reagent was prepared as follows:

$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (2.5% w/v in 3 N H_2SO_4)	-1 vol.
1-amino-2-naphthol-4-sulfonic acid (0.25% w/v)	} aqueous -0.4 vol
NaHSO_3 (15% w/v)	
Na_2SO_3 (0.25% w/v)	
Distilled H_2O	-8.6 vol

Depolymerase activity resulted in the appearance of clear, sharply-defined zones in the blue-white opaque background. If phosphomonoesterase activity was present a marked bluing of the clear zones resulted.

(c) Quantitative Tube Assay

This method was a modification of that described by Reddi (1959).

To 1.5 ml of the substrate solution of DNA (2 mg/ml) was added 1.0 ml of 0.1 M TRIS buffer (pH 8.8) containing 0.04 M MgCl_2 and 0.005 M CaCl_2 , 0.2 ml of 0.025 M MnCl_2 and 0.3 ml of enzyme. A control tube was prepared in the

same manner substituting distilled H_2O for enzyme.

The digest mixture was incubated in a $37^{\circ}C$ H_2O bath for 18 hr. 0.5 ml aliquots were removed at intervals and added to equal volumes of cold 10% TCA or 0.25% uranyl acetate in 10% TCA (UTCA). After 15 min incubation in an ice bath the mixtures were centrifuged in a Servall RC-2 refrigerated centrifuge at 10,000 rpm for 5 min. 0.2 ml of the supernatant was added to 4.8 ml of distilled H_2O and the OD at 260 $m\mu$ measured using a Beckman Model DU Spectrophotometer. One unit of enzyme activity is defined as that amount which causes an increase of 0.1 OD units in the acid-soluble fraction in 18 hr.

The assay required highly polymerized substrate DNA free of low molecular weight acid-soluble material. In these experiments either A grade salmon sperm DNA (Cal-Biochem) or purified C grade DNA was used. The latter was purified in the following manner.

Dry Sephadex G-100 was suspended in H_2O and allowed to stand for 2 or 3 days with intermittent stirring and decantation. The swollen gel was then suspended and washed in 0.14 M NaCl and a 1 x 40 cm column prepared. The C grade DNA was dissolved in 0.14 M NaCl (100 mg/ml) and 1 ml layered on the column. The DNA was eluted with 0.14 M NaCl and 4 ml fractions were collected. An aliquot of each was precipitated with cold 10% TCA and the absorbancy of the acid-soluble fraction at 260 $m\mu$ was measured. Those fractions showing little or no ultraviolet (UV) absorbing material, in the acid-soluble fraction, were pooled and used as substrate and are designated

purified DNA.

EXPERIMENTAL AND RESULTS

1. Effect of pH, Incubation Temperature and Age of Cells upon Enzyme Production

The screening technique described in Materials and Methods was employed. M. sodonensis was inoculated in diametric streaks onto TCS+DNA agar plates at various pH's and incubated at 30° and 37°C for 24 and 48 hr. A positive reaction is demonstrated in Fig. 1. Plate A shows a streak culture of M. sodonensis while Plate B shows the clear zone (indicating nuclease activity) obtained after flooding the same plate with 10% TCA. Table I summarizes the effect of age, temperature and pH on enzyme production.

The results indicate that the enzyme is produced over a fairly wide range of pH and temperature. Maximum cell crop and hence maximum total enzyme production was obtained at pH 7.3 after 48 hr at 30°C and subsequent experiments for recovery employed these growth conditions. Later studies on the purified material showed that the enzyme was inactive at pH 6.0 and, therefore, conclusions concerning production below this pH cannot be made from these data.

2. Detection of Enzyme Activity in Culture Supernatants and Fractions from Column Chromatography

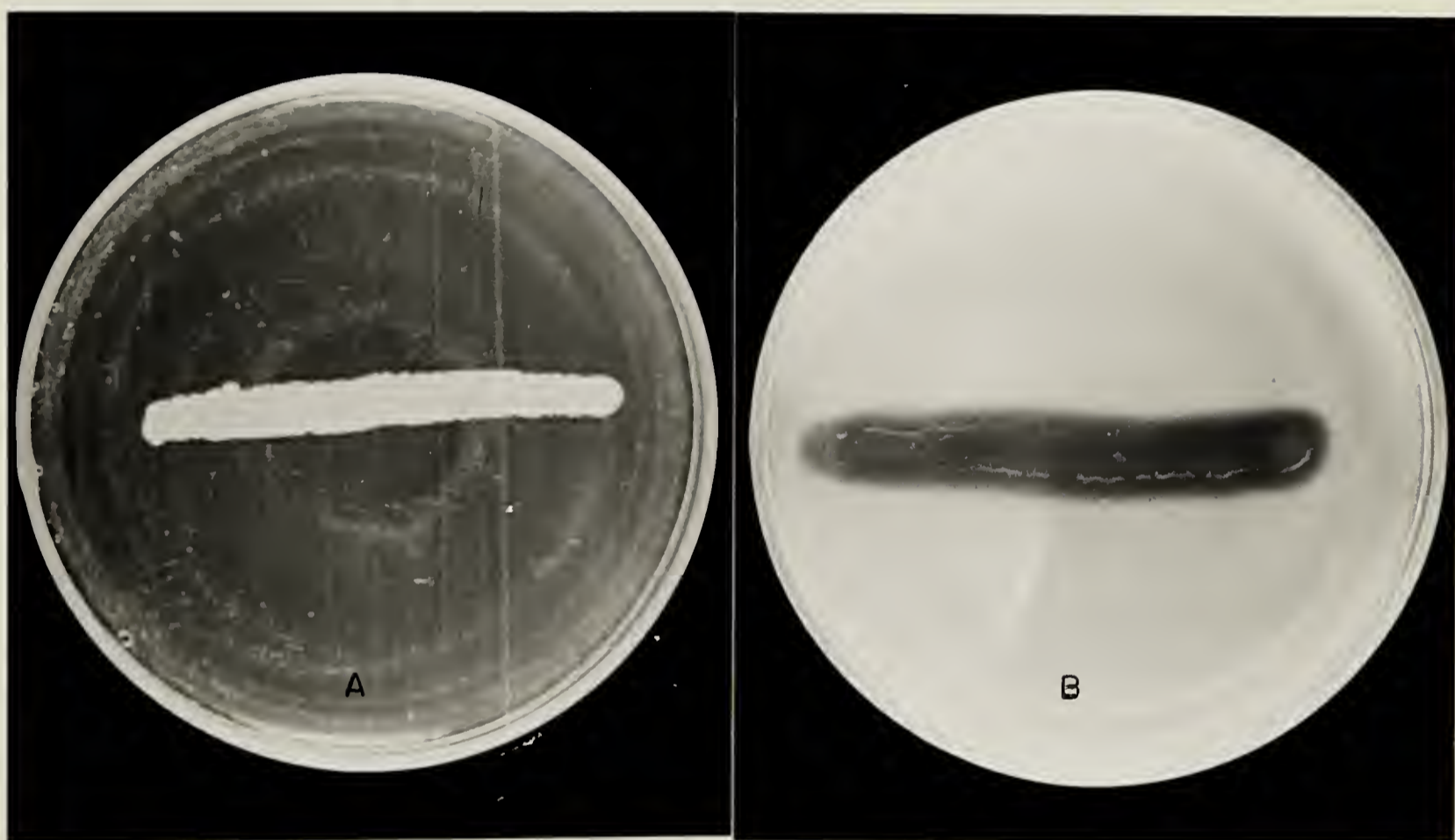
Fig. 2 shows the zones obtained when active fractions were applied to Diffusion Plate Assay Agar. The sensitivity of this technique was compared with that of 2 other developing procedures for plate assay of DNase activity. The results are summarized in Table II.

The sensitivity of the ANS procedure was shown to be greater than that of the other techniques employed and

FIGURE 1

Production of Nuclease by Growing Cultures
of M. sodonensis

Figure 1



- (A) - Streak culture of M. sodonensis growing on TCS agar containing 1 mg/ml DNA.
- (b) - Plate A after flooding with 10% TCA. Growth was removed prior to TCA treatment.

TABLE I

Effect of Age, pH and Temperature on Nuclease

Production by M. sodonensis

Temperature	Incubation Time	pH of Medium	Growth * Response	Zone Size**
30	24 hr	6.0	+++	0
		6.5	++++	0
		7.0	++++	0
		7.5	++++	2
		8.0	++++	3
		8.5	++++	4
	48 hr	6.0	++++	0
		6.5	++++	0
		7.0	++++	2
		7.5	++++	4
		8.0	++++	4
		8.5	++++	4
37°	24 hr	6.0	+++	0
		6.5	++++	0
		7.0	++++	0
		7.5	++++	2
		8.0	++++	4
		8.5	++++	4
	48 hr	6.0	+++	0
		6.5	++++	2
		7.0	++++	2
		7.5	++++	3
		8.0	++++	4
		8.5	++++	4

* Estimated visually.

** Zone size in mm from colony edge to edge of clear zone.

FIGURE 2

Diffusion Plate Assay for Activity on DNA of
Fractions of M. sodonensis Nuclease After
DEAE - Cellulose Chromatography

Figure 2



1,2 and 3- inactive fractions

4,5 and 6- fractions showing DNase activity

0.1 ml aliquots of each fraction were added to antibiotic assay cups embedded in Diffusion Plate Assay Agar and the plate incubated and developed as described in Materials and Methods.

TABLE II

A Comparison of the Sensitivity of Three Developing
Procedures for Plate Assay of DNase Activity

Developing Reagent	Minimum Detectable Enzyme Level (units)
10% TCA + ANS reagent (Berry <u>et al</u> , 1965)	12
0.01% toluidine blue (Streitfeld <u>et al</u> , 1962)	30
10% TCA (DiSalvo, 1958)	48

activity could be demonstrated in fractions in which protein was essentially undetectable either by measuring absorption at 280 m μ or colorimetrically by the technique of Lowry, Rosebrough, Farr and Randall (1951).

3. Effect of Cell Age and Growth Medium on Enzyme Production

Eight flasks each of synthetic medium, with and without NH_4^+ , and of TCS broth were inoculated with 0.1 ml of standard inoculum. The flasks were subjected to vigorous aeration at 30°C and at designated time intervals duplicate flasks of each medium were selected. Ten ml aliquots were removed, centrifuged and the supernatants assayed for enzyme activity by the quantitative tube method. The cells were washed twice and resuspended in distilled H_2O and the turbidity measured at 600 m μ . Dry cell weight was estimated using the standard curve (Fig. 3).

The relationship between cell growth and enzyme production in various culture media and at various ages is shown in Table III.

Both growth and enzyme production were low in the synthetic medium in the absence of NH_4^+ , with no detectable enzyme activity appearing until 36 hr. In the synthetic medium + NH_4^+ and in the TCS broth there was an initial spurt of enzyme production by young, rapidly growing cells following which production fell off and remained stable as the cells entered the stationary phase. This may be seen more clearly from the data as plotted in Fig. 4. Although the total amount of enzyme produced in TCS broth

FIGURE 3

Standard Curve Relating Optical Density
to Dry Cell Weight

FIGURE 3

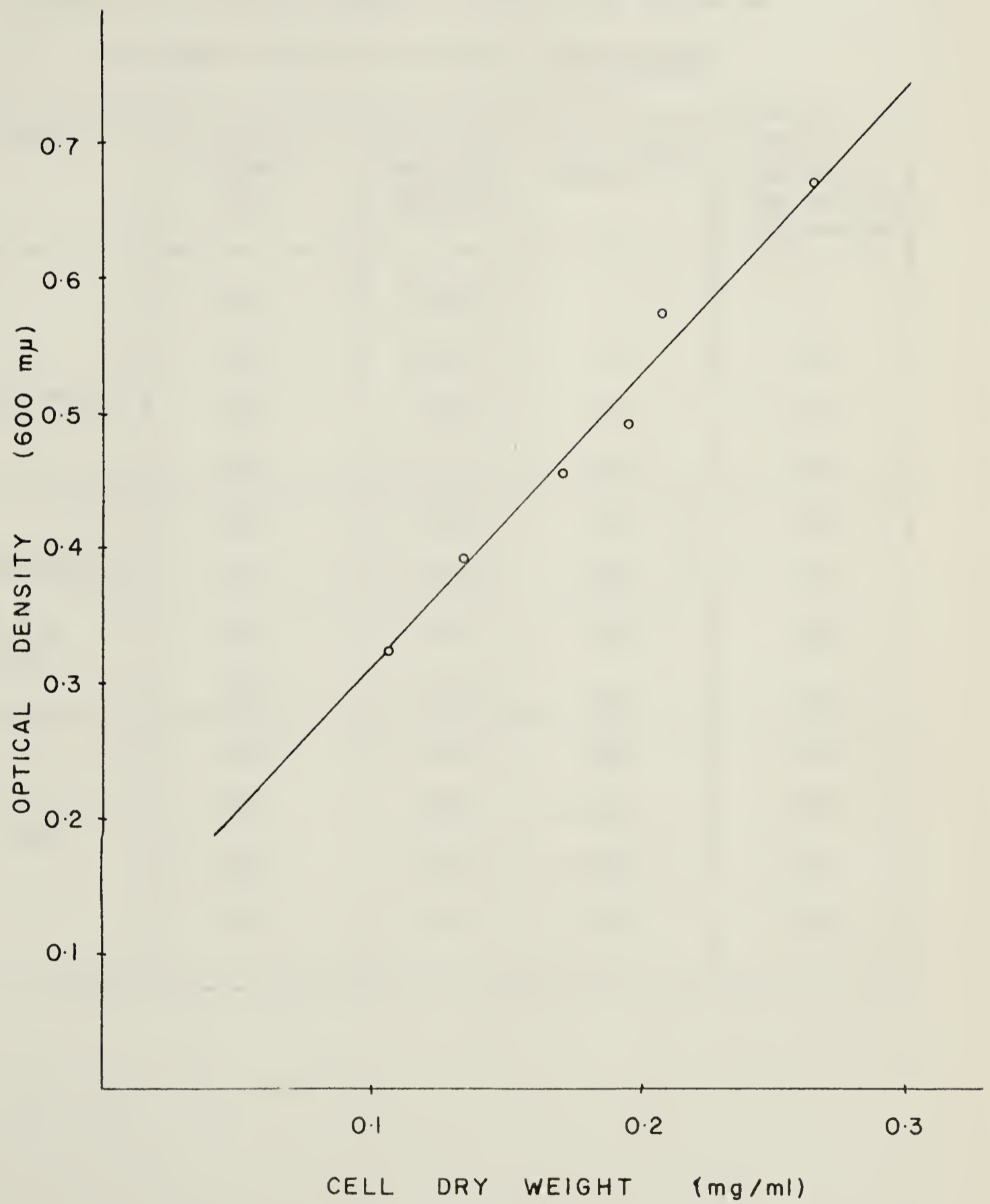


TABLE III

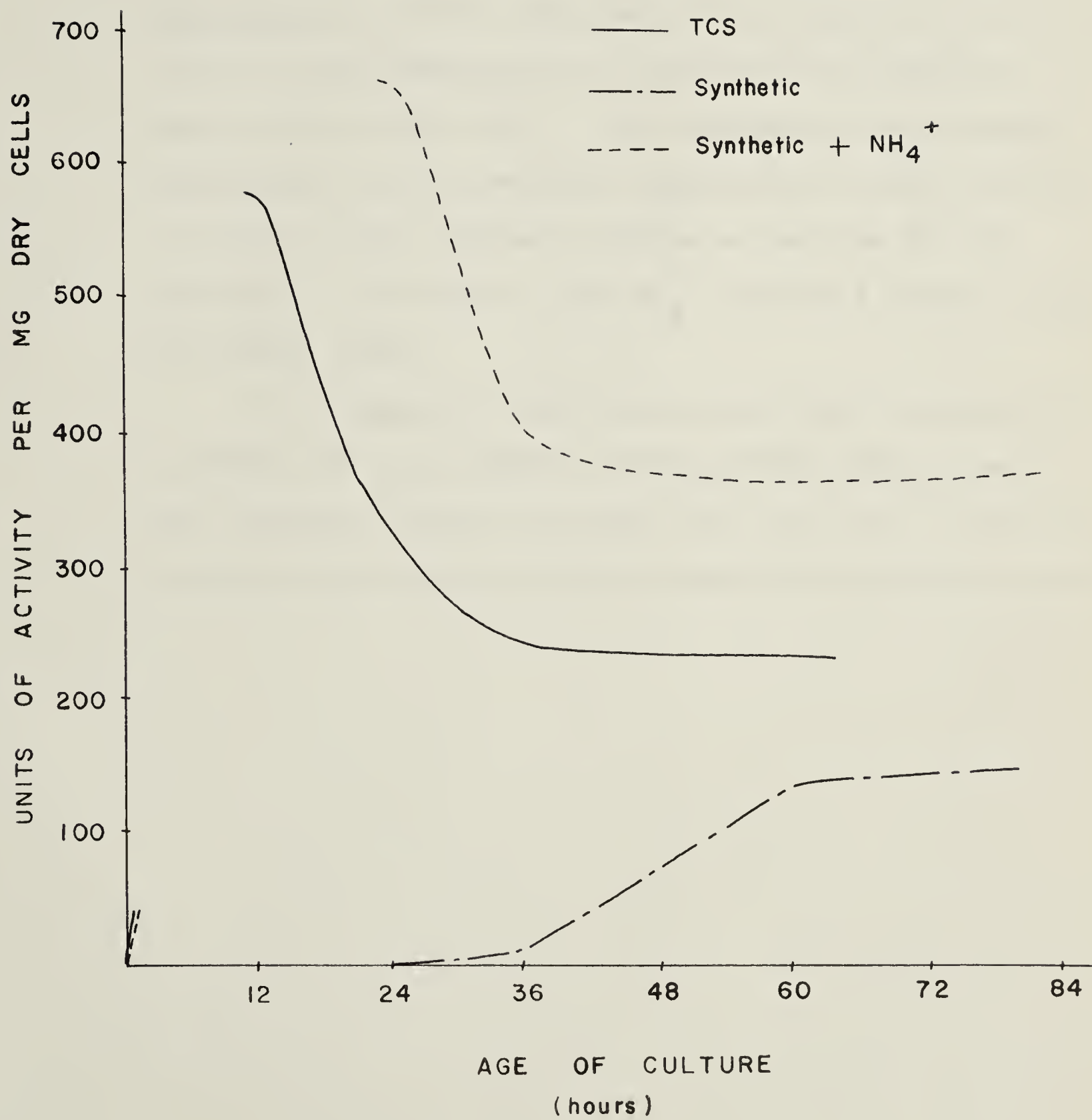
Effect of Culture Medium and Age of Cells on
Nuclease Production by M. sodonensis

Medium	Incubation Time (hr)	Cell Dry Weight (mg/ml)	Activity (units/ml)	Activity (units/mg dry weight of cells)
Synthetic	24	0.360	0	0
	36	0.800	10	12
	60	1.600	210	131
	84	2.134	329	154
Synthetic + NH_4^+	24	1.164	775	666
	36	2.240	925	385
	60	2.560	925	361
	84	2.360	925	392
TCS	12	1.190	688	578
	24	3.840	1143	298
	36	6.420	1500	234
	60	6.192	1463	236

FIGURE 4

Effect of Culture Medium and Age of Cells on
Nuclease Production by M. sodonensis

FIGURE 4



was greater than in the synthetic medium + NH_4^+ , the amount of enzyme per unit cell was comparable. NH_4^+ was essential for enzyme production and little or none was formed in its absence. Campbell et al (1961a) showed that NH_4^+ was a specific growth requirement for M. sodonensis and if not supplied in the medium it was obtained by the deamination of glutamic acid (yielding α ketoglutaric acid + NH_3). This deamination is probably responsible for the eventual appearance of small amounts of enzyme in the synthetic medium as well as for the abundance in TCS which lacks NH_4^+ but has a large variety of α -amino acids.

It is apparant from the results that the enzyme is a product of young rapidly growing cells rather than of cell autolysis since the amount per unit cell is highest in young cells where autolysis would not yet have occurred.

PART II

ISOLATION AND PURIFICATION OF ENZYME ACTIVITY

PART II

Materials and Methods

1. Precipitation

$(\text{NH}_4)_2\text{SO}_4$ was added gradually to the supernatant of a 48 hr fluid culture of M. sodonensis to yield the desired saturation. The precipitation was carried out at 4°C and the resultant solution stored at this temperature for 16 hr. The precipitate was harvested by centrifugation, in a Servall RC-2 refrigerated centrifuge, and dissolved in 0.01 M TRIS buffer pH 8.8. The solution was then dialysed for 16 hr at 4°C in 0.01 M TRIS buffer pH 8.8 which was 0.004 M with respect to Mg^{++} and Ca^{++} .

2. Chromatography

(a) Preparation of Columns

(i) DEAE-cellulose (Eastman Kodak) was suspended in distilled H_2O and the fines decanted. The slurry was washed with 0.5 N NaOH until no further color was removed then washed with distilled H_2O to a neutral pH. An equal volume of 95% ethanol was washed through, followed by excess distilled H_2O to remove the ethanol. 0.001 M versene (pH 7) was then passed through to remove any heavy metals, followed by distilled H_2O to remove the versene. A final wash with 0.5 N NaOH followed by distilled H_2O , until the eluant attained a neutral pH, completed the cycle. The cellulose was then suspended in M TRIS buffer pH 8.8 and stored at 4°C until used.

1 x 10 cm columns were prepared and equilibrated

with 0.01 M TRIS buffer pH 8.8. When large amounts of enzyme were required, 5 x 20 cm columns were prepared.

Five ml of the dialysed active $(\text{NH}_4)_2\text{SO}_4$ fraction (containing about 4 mg of protein) were applied to the column and the column washed with 30 ml of 0.01 M TRIS buffer pH 8.8. The remaining protein was eluted from the column with an NaCl gradient of 0 - 0.5 M NaCl in 0.01 M TRIS buffer pH 8.8. Four ml fractions were collected and assayed for salt concentration, protein and diesterase and monoesterase activity.

(ii) Sephadex G-100 columns were prepared as described in Part I. Active fractions from DEAE-cellulose were pooled, concentrated, dialysed and rechromatographed on a 1 x 40 cm Sephadex G-100 column. A column of these dimensions had a void volume of 11 ml and the flow rate was 40 ml/hr. The protein was eluted with 0.01 M TRIS buffer pH 8.8 and 4 ml fractions were collected and assayed for protein and enzyme activity.

(b) Analysis of Fractions

(i) Protein content of the various fraction was determined by measuring OD at 280 m μ or by the colorimetric method of Lowry et al (1951) which was conducted as follows:

Reagent A:- 2% Na_2CO_3 in 0.1 N NaOH

Reagent B:- 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% potassium tartrate

Reagent C:- 50 ml of A + 1 ml of B (freshly prepared)

Reagent D:- Folin-Ciocalteu Phenol Reagent (BDH)

diluted 1:2 with H_2O .

To 0.5 ml of protein solution were added 2.5 ml of reagent C. After 10 min incubation at room temperature 0.25 ml of reagent D was added. Solutions were incubated for 30 min at room temperature and the OD's read at 500 mμ. A control tube, containing H₂O in place of the protein solution, was set up in the same manner. A standard curve was prepared (Fig. 5) using known concentrations of bovine serum albumin as the standard protein solution.

(ii) Phosphomonoesterase activity was determined by measuring the release of inorganic phosphate (P_i) (Fiske and Subbarow, 1925).

Molybdic Acid Reagent:

(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	25 gm
10 N H ₂ SO ₄	300 ml

Diluted to 1000 ml with distilled H₂O

Aminonaphtholsulfonic Acid Reagent:

NaHSO ₃	15.0 gm
Na ₂ SO ₃	0.25 "
1-amino-2-naphthol-4-sulfonic acid	0.25 "
Distilled H ₂ O	100 ml

A 1 ml aliquot of digest was treated with 1 ml of cold 10% TCA, the precipitate removed and the sample diluted to 5 ml with distilled H₂O. To the 5 ml sample were added 1 ml of molybdic acid reagent, 0.4 ml of aminonaphtholsulfonic acid reagent and 3.6 ml of distilled H₂O. After 10 min at room temperature the OD's at 660 mμ were read and the concentration of P_i determined from the

FIGURE 5

Standard Curve for Protein Determination by the
Method of Lowry et al (1951)

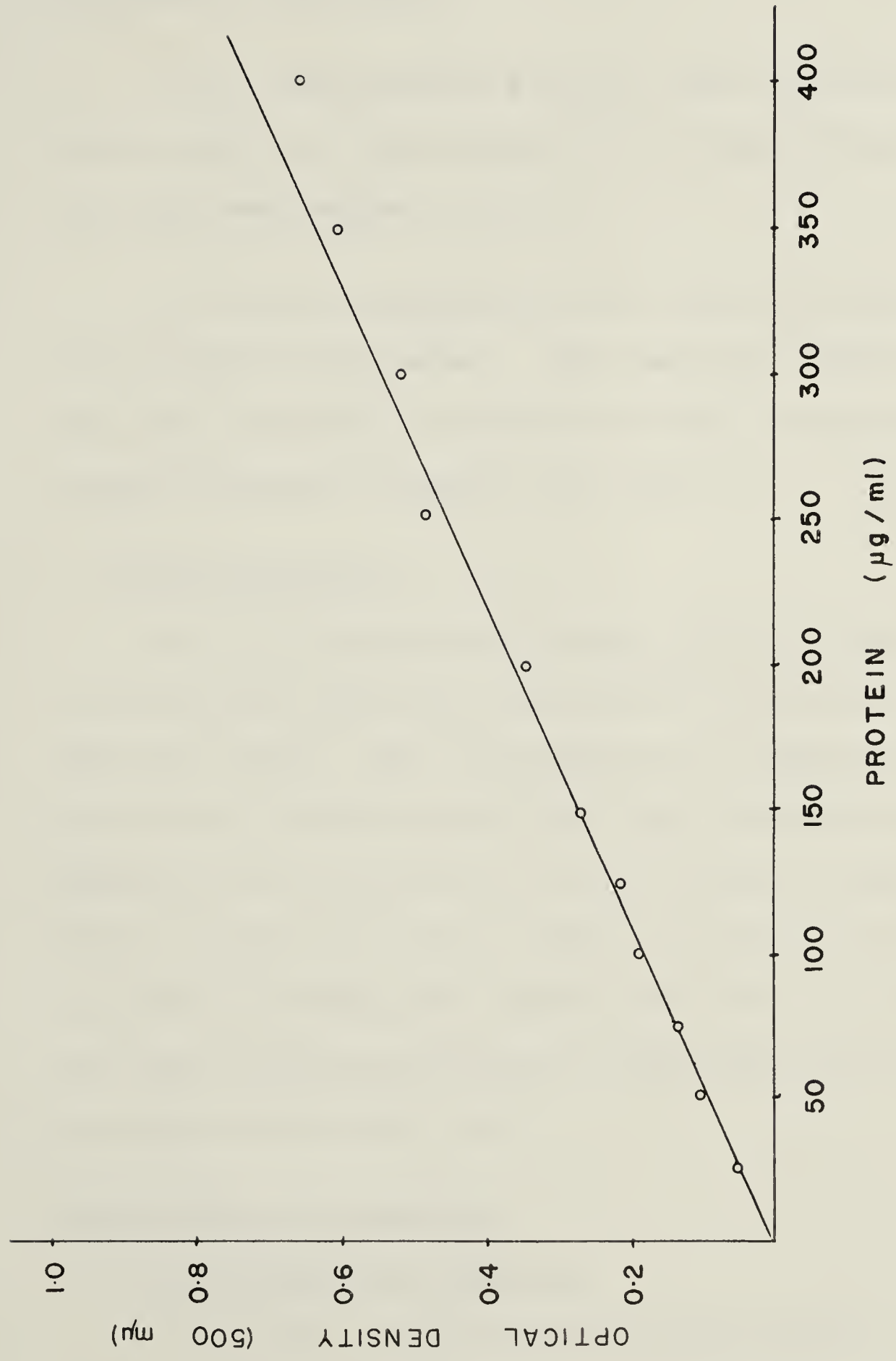


FIGURE 5

standard curve (Fig. 6). One unit of activity is defined as that amount of enzyme releasing $1 \mu\text{M}$ of P_i under the experimental conditions.

(iii) Depolymerase activity was measured by the diffusion plate and quantitative tube assays described in Materials and Methods, Part I.

(iv) Salt concentration was measured using a Radiometer Conductivity Meter. The measured conductivity values were converted to molarity of NaCl by means of a previously prepared standard curve (Fig. 7).

3. Electrophoresis

50 λ of the purified enzyme solution were streaked on duplicate Schleicher and Schüll filter paper electrophoresis strips. The electrophoretic separation was carried out using an LKB 3276B Paper Electrophoresis Apparatus at 200 volts for 10 hr in 0.125 M Veronal buffer pH 8.6. One strip was stained for protein using 0.002% nigrosin in 2% CH_3COOH (Smith 1960) for 16 hr while the duplicate strip was assayed for depolymerase activity on Diffusion Plate Assay Agar.

EXPERIMENTAL AND RESULTS

1. Precipitation of Activity

$(\text{NH}_4)_2\text{SO}_4$ precipitation was carried out in a step-wise manner and 98% of the precipitable activity was contained in the 0.4 - 0.55 saturated fraction. Increasing the concentration to 0.75 saturation precipitated the re-

FIGURE 6

Standard Curve for the Determination of
Inorganic Phosphate According to the
Method of Fiske and Subbarow (1925)

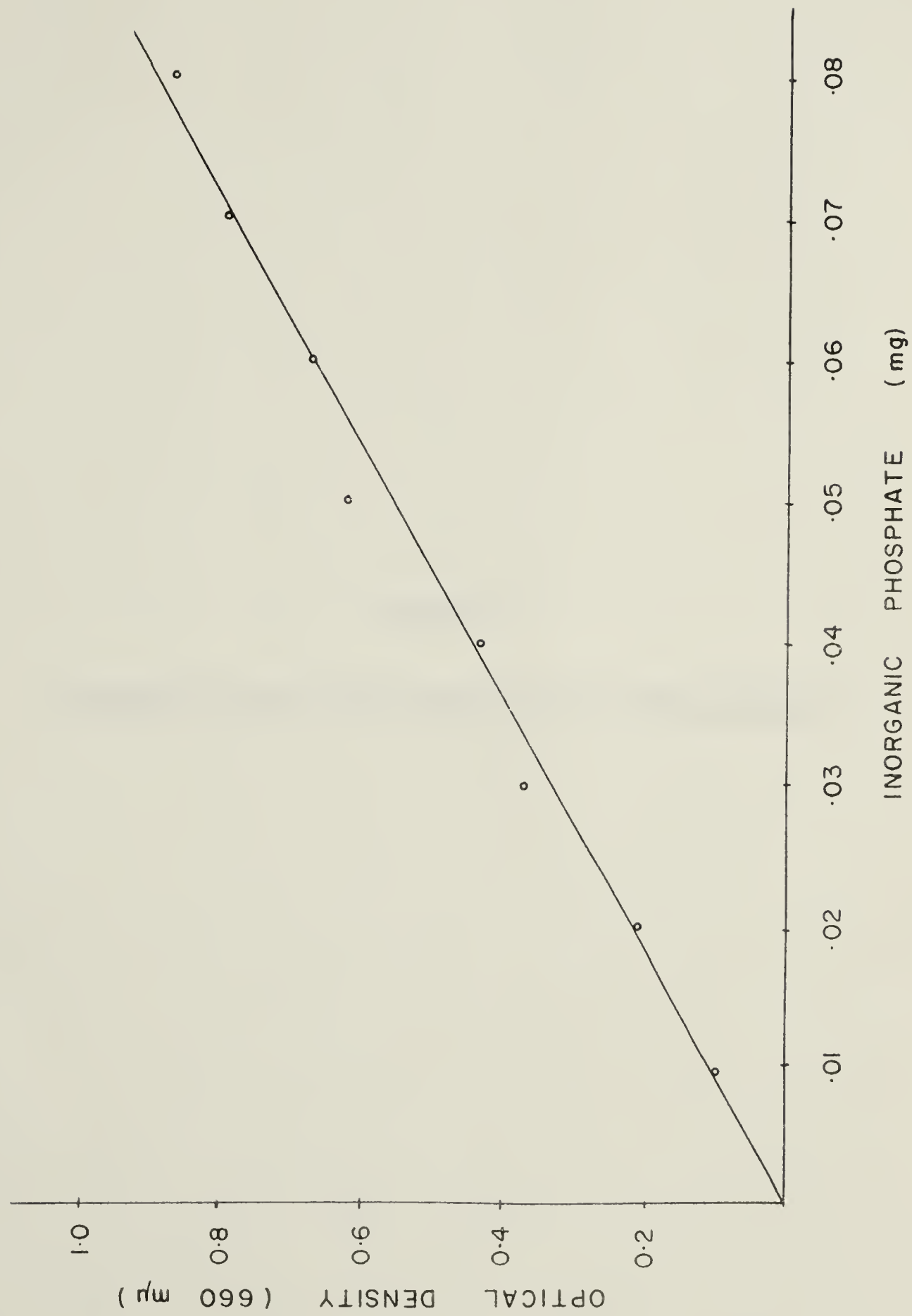


FIGURE 6

FIGURE 7

Standard Curve for Conductivity Measurements

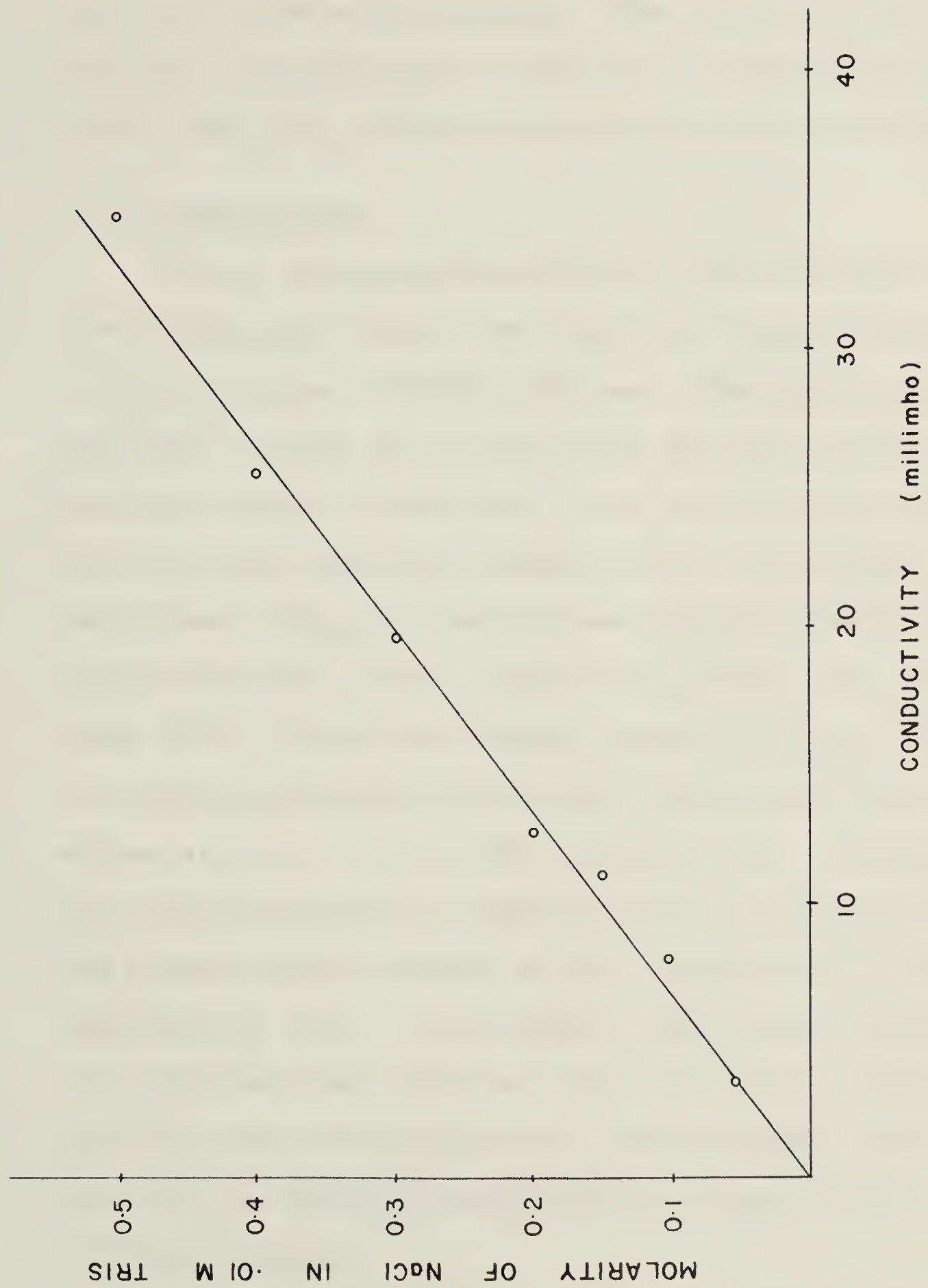


FIGURE 7

maintaining 2% of the activity but greatly increased the amount of contaminating protein. The results of this experiment are summarized in Table IV. Controlling the pH during $(\text{NH}_4)_2\text{SO}_4$ precipitation did not alter the results.

2. Chromatography

Fig. 8 is the elution pattern obtained when a dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to and eluted from a 1 x 10 cm column of DEAE cellulose. The first protein peak was not held up on the column but was carried through with the initial buffer wash. All activity was associated with the peak appearing between 0.15 - 0.25 M NaCl. If an undialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was chromatographed, essentially the same elution pattern of protein was evident but there were 2 fractions showing enzyme activity, one with the first unadsorbed protein peak (Fraction I) and another eluted at 0.15 - 0.25 M NaCl (Fraction II). Dialysis and rechromatography on DEAE-cellulose of Fraction I gave the protein elution shown in Fig. 9 with all the activity appearing at 0.15 - 0.25 M NaCl. From these results it was concluded that Fraction I was an artifact caused by the high salt concentration in the undialysed material and that the DEAE-cellulose chromatography yields only 1 active fraction.

The pooled active fractions from DEAE-cellulose were passed through Sephadex G-100 using 0.01 M TRIS buffer pH 8.8 as the eluant. The results are shown in Fig. 10. There was one peak of activity appearing immediately after the void volume was collected and the

TABLE IV

Precipitation of Nuclease Activity of

M. sodonensis with $(\text{NH}_4)_2\text{SO}_4$

Saturation of $(\text{NH}_4)_2\text{SO}_4$	Total Enzyme Recovered (units)
0 - 0.4	0
0.4 - 0.55	1.75×10^5
0.55- 0.75	1.4×10^3

FIGURE 8

Chromatographic Separation on DEAE-cellulose
of $(\text{NH}_4)_2\text{SO}_4$ Fractionated *M. sodonensis* Nuclease

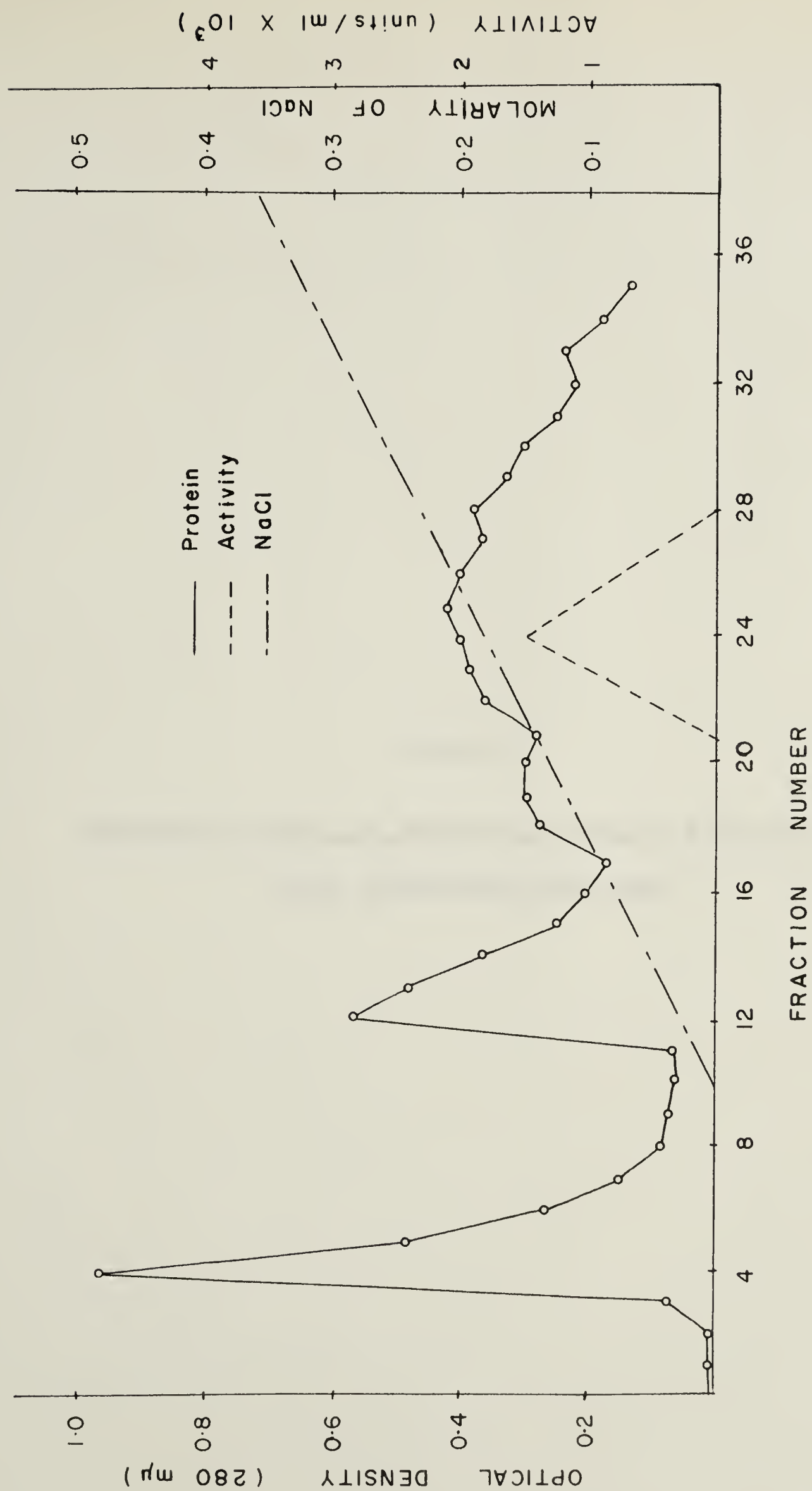


FIGURE 8

FIGURE 9

Rechromatography on DEAE-cellulose of Fraction I
of M. sodonensis Nuclease

FIGURE 9

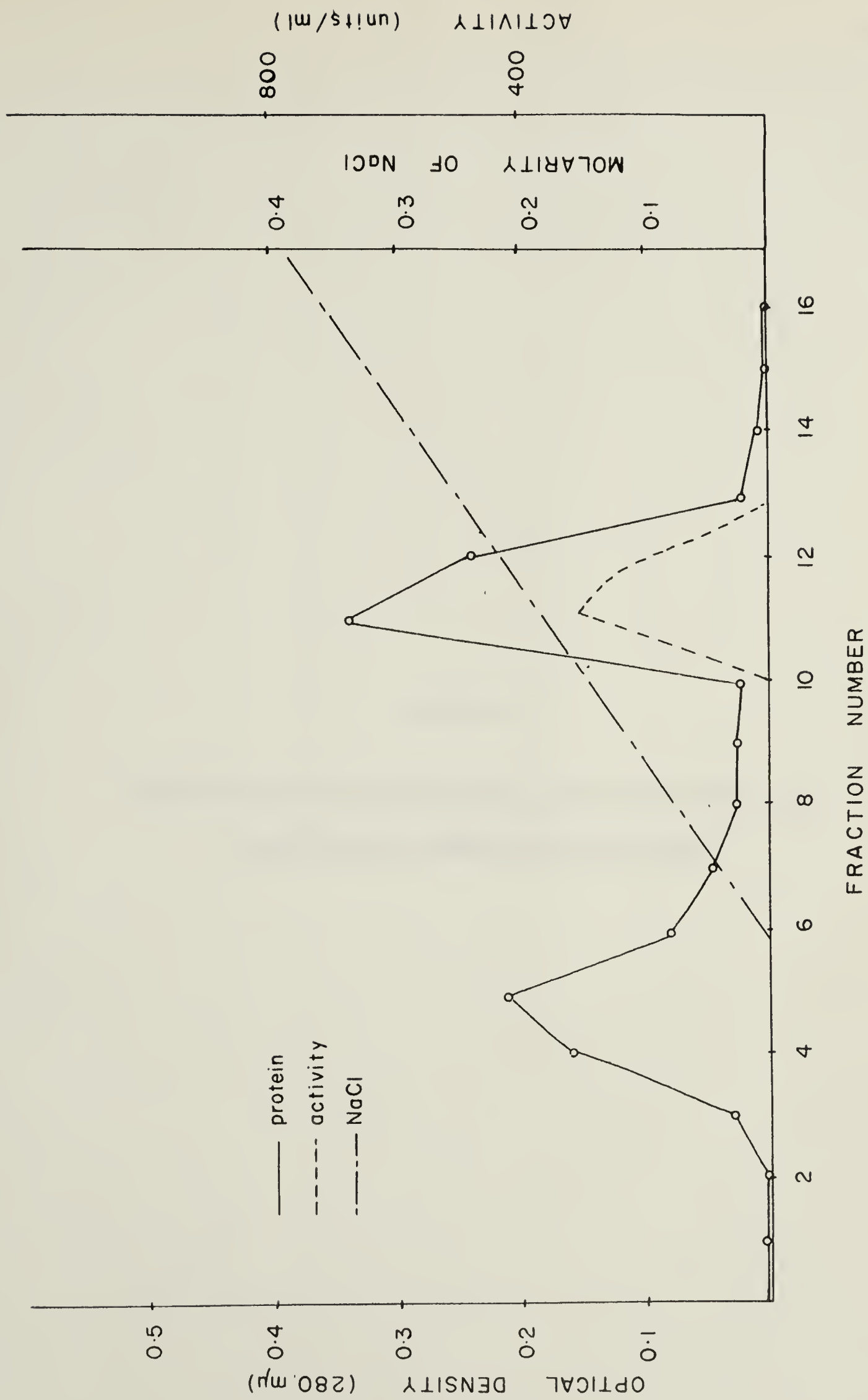


FIGURE 10

Gel Filtration on Sephadex G-100 of Partially
Purified M. sodonensis Nuclease

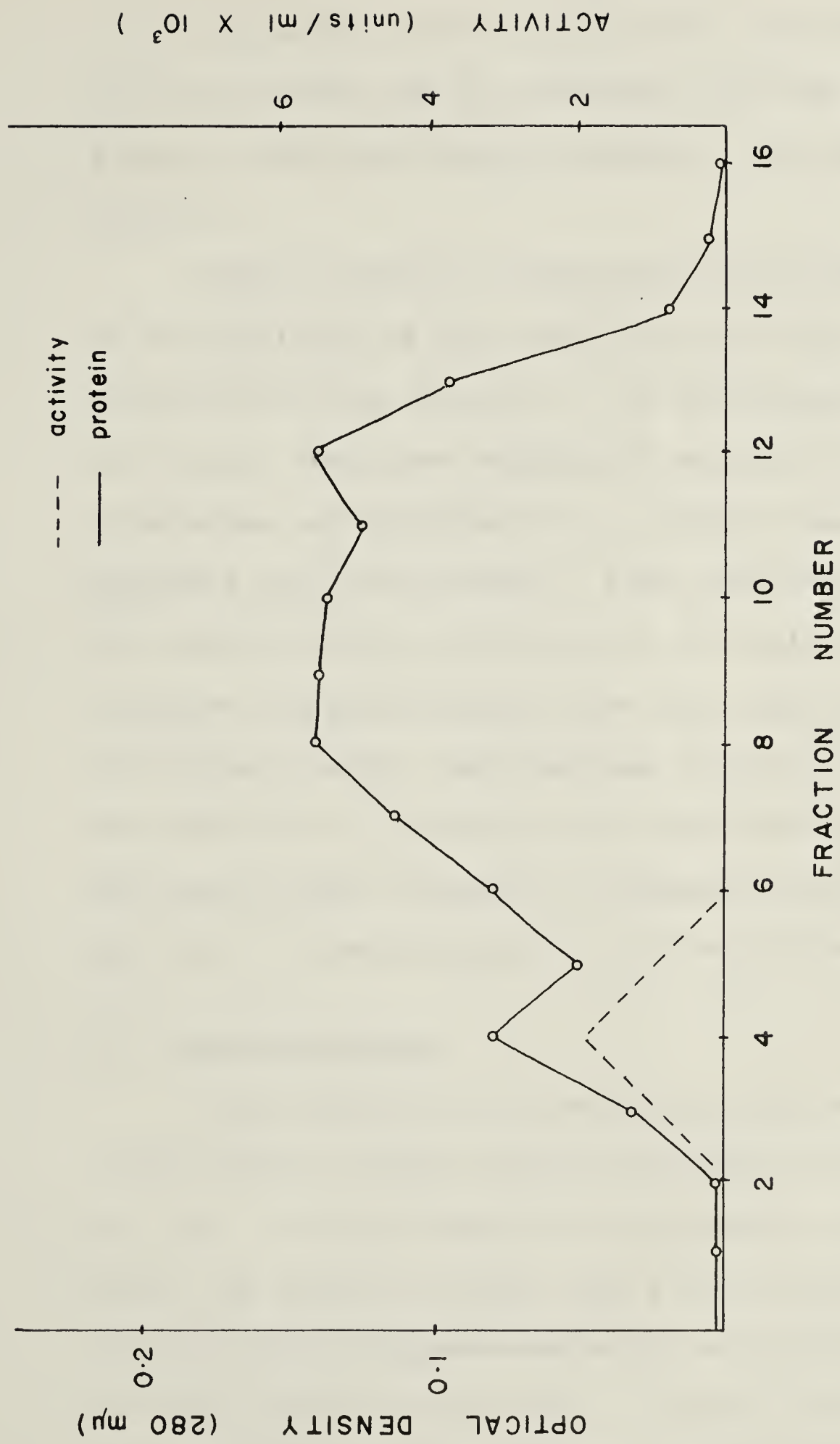


FIGURE 10

later appearing protein peaks were inactive. The degree of purification and the recovery obtained at the various steps of the purification procedure are summarized in Table V.

Final recovery of depolymerase activity was 42.6% of the activity of the crude material and a 1400 fold purification was obtained. The phosphomonoesterase activity in all cases was associated entirely with the phosphodiesterase activity and in no instance was it possible to separate the 2 activities. Both diesterase and monoesterase activities were purified to the same extent if one compares the purification from the $(\text{NH}_4)_2\text{SO}_4$ fractionation step onwards (depolymerase 192 fold, phosphomonoesterase 186 fold). The high value obtained in the analysis of the supernatant fraction for monoesterase activity is probably due to contaminating P_i in the medium.

3. Electrophoresis

The results of an electrophoretic analysis of the enzyme after passage through Sephadex G-100 is shown in Fig. 11. A single band of depolymerase activity can be seen 7 cm from the origin and a bluing of this zone indicated that phosphomonoesterase activity was restricted to this single zone as well. Protein could barely be detected on the stained strip, the concentration of protein being just at or below the sensitivity of the stain.

TABLE V

Purification and Recovery at the Various Steps
of Purification of M. sodonensis Nuclease

Fraction	Phosphomono- esterase S.A.*	Depoly- merase S.A.	Total Depoly- merase Activ- ity (units)
Crude Culture Supernatant	9.7	300	1.13×10^6
0.55 Saturated (NH ₄) ₂ SO ₄ Pre- cipitate	26.4	2210	5.48×10^5
Dialysis	42	4680	5.48×10^5
DEAE-cellulose Chromatography	566	51,300	5.48×10^5
Sephadex G-100 Gel Filtration	4900	425,000	4.82×10^5

* Specific activity in units/mg protein.

FIGURE 11

Electrophoretic Analysis of Purified
M. sodonensis Nuclease

Figure 11



0 - Origin

Diffusion Plate Assay Agar was developed as described in Materials and Methods, Part I. The clear zone indicates depolymerase activity.

PART III

CHARACTERIZATION AND MODE OF ACTION

PART III

MATERIALS AND METHODS

1. Substrates Employed

A grade salmon sperm DNA (1 mg/ml) was used in all assays for depolymerase activity. Denatured DNA was prepared by heating at 100°C for 10 min followed by rapid cooling. The Poly A (0.5 mg/ml) used in the substrate specificity study was obtained from Mann Biochemical. C grade yeast RNA - 2.5 mg/ml (CalBiochem) was purified as follows: (Eaves et al, 1963)

The RNA was put into solution by the dropwise addition of 3.5% NaHCO₃. The solution was precipitated with 3 volumes of cold 95% ethanol which had been adjusted to pH 4.5 with dilute acetic acid. The precipitate was harvested by centrifugation, dissolved in the original volume of distilled H₂O and dialysed with constant stirring for 48 hr against repeated changes of distilled H₂O. The RNA content of the dialysate was determined by dry weight measurements. The purified RNA solution was diluted to a concentration of 10 mg/ml and stored at -20°C.

5' adenylic acid (pA) was used in all assays for phosphomonoesterase activity. Specificity studies employed the 3' and 5' ribomononucleotides, and the 5' deoxyribonucleotides of adenine, guanine, cytosine, uracil and thymine (Ap,pA,dpA,Gp,pG,dpG,Cp,pC,dpC,Up,pU,pT) as well as thymidine-3',5'-diphosphate (pTp), adenosine-5'-diphosphate (ADP) and adenosine-5'-triphosphate (ATP). All of the nucleotides employed were obtained from CalBiochem.

Concentration of nucleotides in the assay mixture was 0.5 mg/ml.

2. Heat Inactivation Studies

0.5 ml aliquots of the purified enzyme preparation were placed in 16 x 150 mm pyrex test tubes and heated in a water bath at the indicated times and temperatures. Samples were removed and immediately chilled in an ice bath and assayed for depolymerase and phosphomonoesterase activity as previously described.

3. pH Dependency Studies

Standard reaction mixtures were prepared employing the following buffers to provide the indicated pH values. (a) pH 5 and 6 - 0.1 M phosphate buffer was prepared by adding 0.1 M KH_2PO_4 to 0.1 M Na_2HPO_4 until the desired pH was obtained. (b) pH 7, 8.7 and 9 - 0.1 M TRIS buffer (pH adjusted with HCl). (c) pH 10 and 11 - 0.1 M Borate buffer - an equimolar solution of H_3BO_3 and KCl was prepared and the pH adjusted with 0.2 M NaOH.

4. Cation Activation

Fresh bottles of reagent grade chemicals and dichromate cleaned glassware were used throughout these studies. Reaction mixtures were prepared containing varying concentrations of the designated cations, and enzyme activity was determined.

5. Exonuclease and Endonuclease Activity

Pancreatic DNase (Worthington) and Snake Venom Phosphodiesterase (Dr. B.G. Lane) were used as controls for

endo and exonucleolytic activity.

Two methods were employed:

(a) TCA vs. UTCA precipitation (Helleiner, 1955) - duplicate aliquots of the standard reaction mixtures were removed at the designated time intervals and precipitated with cold 10% TCA and UTCA. The supernatants were examined spectrophotometrically at 260 mμ for the appearance of acid-soluble, UV-absorbing products.

(b) Viscometry - 5 ml aliquots of a standard reaction mixture were removed at the designated time intervals and added to 0.5 ml of 0.2 M versene to stop the reaction. 5 ml of sample were drawn into a 5 ml Ostwald viscometer and placed in a 20°C H₂O bath. After equilibration the flow times were measured and the relative viscosities determined. 0.1 M TRIS buffer was the solvent used in the experiments.

6. Analysis of Reaction Products

Snake Venom Phosphodiesterase and Spleen Phosphodiesterase (Sigma) were used as the control enzymes in this study.

Reaction mixtures were digested for 24 hr and chromatographed using the method of Singh and Lane (1964). Whatman #1 filter paper was impregnated with a mixture of 1 volume of saturated (NH₄)₂SO₄ and 9 volumes of distilled H₂O. The developing solvent was 75% ethanol. The strips were developed for 18 hr and the spots located by scanning the strip with a hand UV lamp.

The digest was analysed for the presence of P_i.

7. Sedimentation Studies

Analyses were made using a Spinco Model E Analytical Centrifuge employing Schlieren optics. Protein concentration (Lowry et al, 1951) was 0.1% in TRIS buffer (ionic strength 0.1).

The following conditions were employed:

(a) Sedimentation velocity experiments

Rotor speed - 59,780 rpm

Bar angle - 50° and 75°

Rotor temperature - 19.2°C

(b) Sedimentation equilibrium

Rotor speed - 12,590

Bar angle - 50°

Rotor temperature - 19.2°C

Photographs were taken at 16 min intervals. The time at which the rotor reached the desired speed was designated time "0".

EXPERIMENTAL AND RESULTS

1. Heat Inactivation

Fig. 12a shows the results of an experiment in which purified enzyme (400 units/ml in TRIS buffer pH 8.8) was heated for 5 min at various temperatures. Heating at 45°C had little or no effect upon either the depolymerase or phosphomonoesterase activity. After 5 min at 50°C , 34% of the depolymerase activity was destroyed while phosphomonoesterase activity remained at 88% of the unheated enzyme. Depolymerase activity was completely destroyed after 5 min at 55°C while phosphomonoesterase was inactivated after 5

FIGURE 12

Effect of Heat on Activity of Purified M.
sodonensis Nuclease

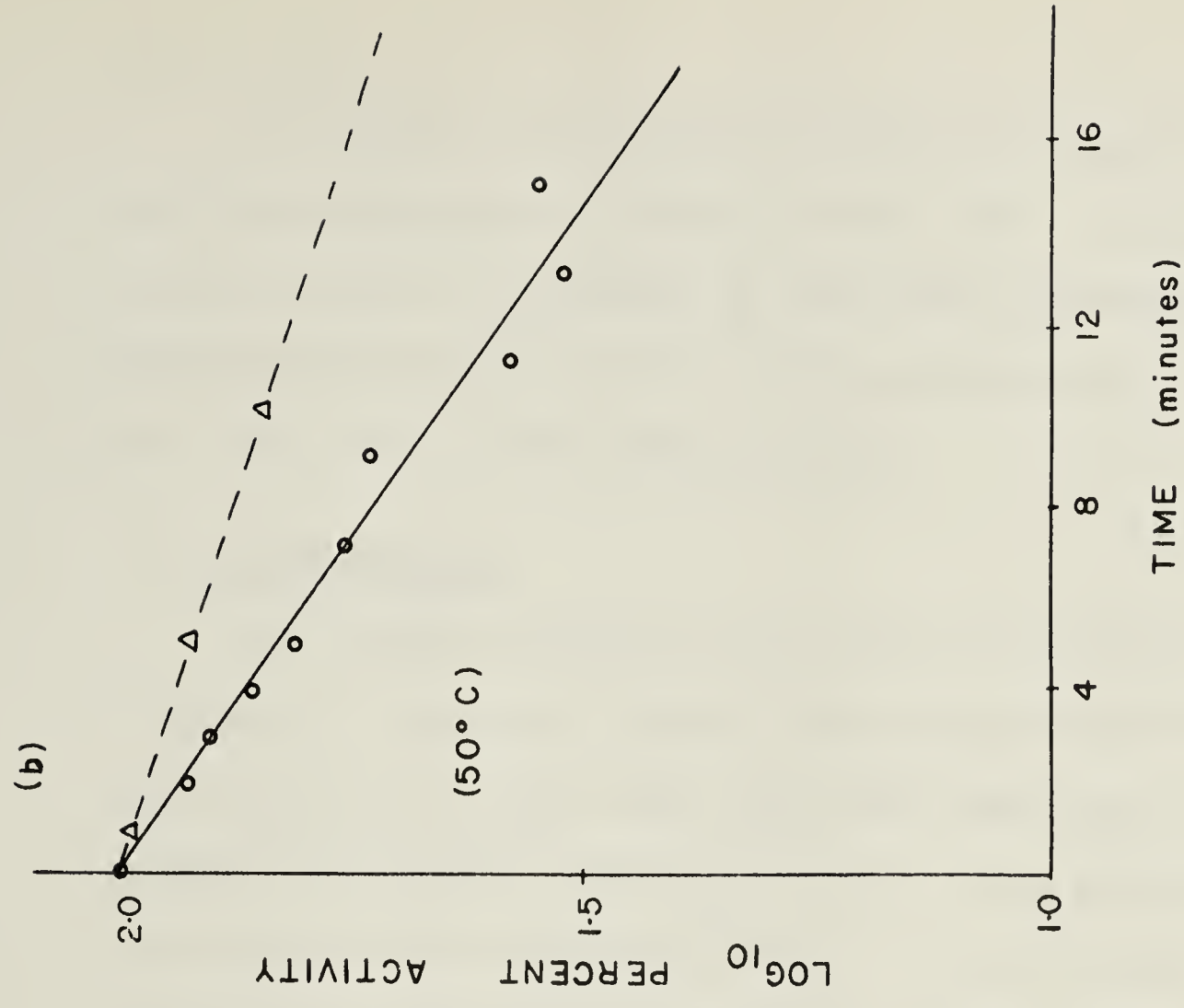
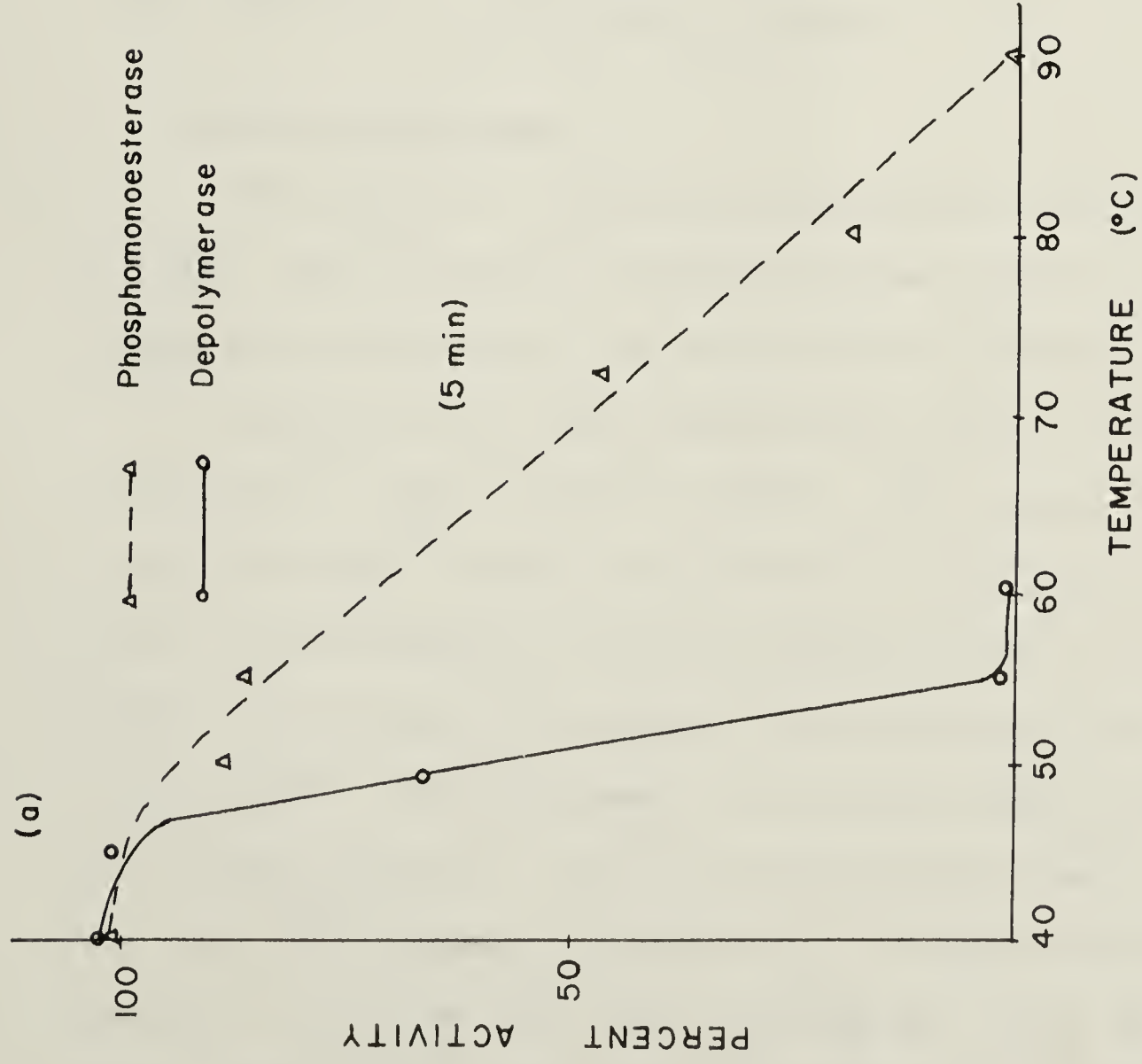


FIGURE 12

min at 90°C.

The effect of heating the purified enzyme for various time intervals is shown in Fig. 12b. A linear inactivation curve is obtained on the semilogarithmic plot. Phosphomonoesterase inactivation proceeds at a slower rate than does that of the depolymerase.

2. pH Dependence

The effect of varying the pH of the reaction mixture is shown in Fig. 13. Optimal depolymerase activity occurred from pH 8.2-9 with less than 40% remaining at pH 11 and a complete loss of activity at pH 6. The phosphomonoesterase activity was measured from pH 7 to pH 10 and followed essentially the same pH dependence curve. pH 8.8 has been used routinely in the assay system.

3. Cation Activation

The effect of various combinations and concentrations of Mg^{++} , Mn^{++} and Ca^{++} on depolymerase activity was investigated and the results are summarized in Table VI.

None of the 3 ions alone would support activity nor would $Ca^{++} + Mn^{++}$ or $Ca^{++} + Mg^{++}$. The slight stimulation noted with Mg^{++} alone and with $Ca^{++} + Mg^{++}$ was probably due to the presence of trace amounts of Mn^{++} in the $MgCl_2$ since no further stimulation occurred until the concentration of Mn^{++} was increased. Similarly there is some activity noted with $Mn^{++} + Ca^{++}$ but here again there is as much as 0.5% Mg^{++} present in the $CaCl_2$. A concentration of 0.04 mM Mg^{++} , 0.005 mM Mn^{++} and 0.005 mM Ca^{++} gave optimum activity. 65% activity was obtained with 0.04 mM Mg^{++} and 0.005

FIGURE 13

Effect of pH on Activity of Purified M.
sodonensis Nuclease

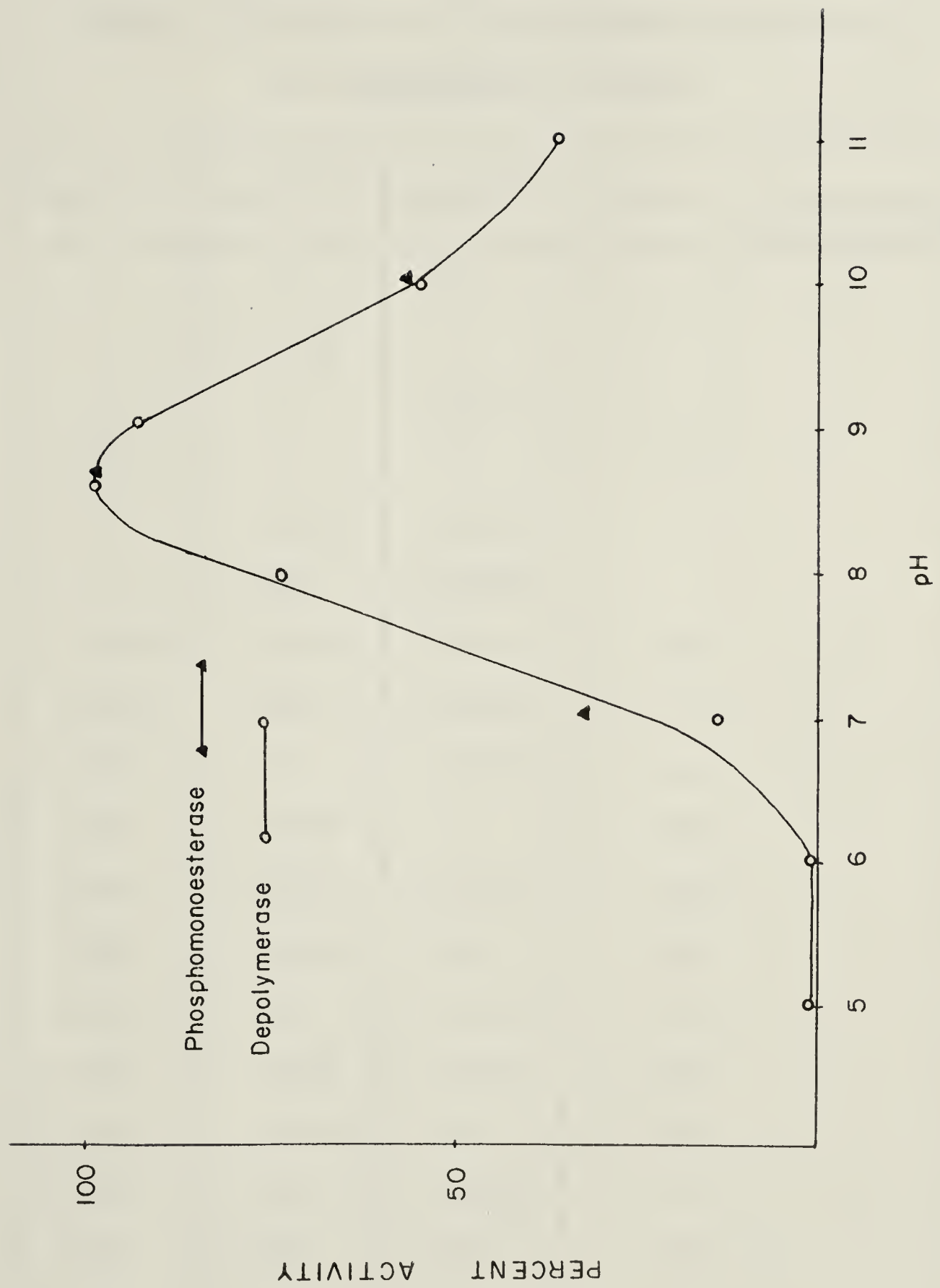


FIGURE 13

TABLE VI

Effect of Cations on Depolymerase Activity of
M. sodonensis Nuclease

$[Mg^{++}]^*$	$[Mn^{++}]^*$	$[Ca^{++}]^*$	Activity (units/ml) of enzyme**
0	0	0	0
0	0.005	0	0
0	0	0.04	0
0.04	0	0	50
0	0.005	0.04	70
0	0.04	0.005	0
0.005	0.005	0.04	80
0.005	0.04	0.005	0
0.04	0	0.005	52
0.04	0.005	0	115
0.04	0.005	0.005	178
0.04	0.005	0.01	165
0.04	0.01	0.005	52
0.06	0.005	0.005	187
0.06	0.005	0.01	182
0.06	0.01	0.005	70
0.06	0.01	0.01	52
0.06	0.015	0.015	27

* Concentration in mm./3 ml of assay mixture.

** Calculated. 3 ml of assay mixture contains 0.3 ml of enzyme.

mM Mn^{++} and the addition of Ca^{++} had a synergistic effect. An increase in Mg^{++} over 0.04 mM gave only a slight increase in activity and increasing Ca^{++} above 0.005 mM had no effect. The concentration of Mn^{++} was critical and any increase in the concentration over 0.005 mM was inhibitory.

The effect of cations on phosphomonoesterase activity is summarized in Table VII.

Here it is apparent that Mn^{++} alone would support activity and Ca^{++} and Mg^{++} were not required. As with the depolymerase system a concentration of 0.005 mM Mn^{++} was optimum and any increase over this level resulted in inhibition of the activity.

4. Substrate Specificity

Depolymerase activity was tested against RNA and Poly A as well as denatured and native DNA. Fig. 14 shows activity against Poly A as detected by the Diffusion Plate Assay. As seen in Fig. 15 the enzyme was active on all 3 of the substrates tested, although the rate of attack on RNA was slightly slower than on DNA. There was a lag of slightly more than 30 min with denatured DNA and of 2 hr with native DNA, but after the lag period the rates were parallel. The longer lag period with native DNA could be eliminated by preincubation of the assay mixture for 4 hr at 37°C before the addition of the enzyme. This treatment did not denature the DNA since there was no hyperchromic effect.

Specificity of the phosphomonoesterase activity was investigated using the various nucleotides. As shown in

TABLE VII

Effect of Cations on Phosphomonoesterase

Activity of M. sodonensis Nuclease

$[\text{Mg}^{++}]^*$	$[\text{Mn}^{++}]^*$	$[\text{Ca}^{++}]^*$	Activity units/ml of enzyme**
0	0	0	0
0	0	0.04	0
0.04	0	0	0
0	0.04	0	0
0	0.005	0	110
0.04	0	0.005	0
0.04	0.005	0.005	95
0.04	0.01	0.005	5
0.04	0.005	0	95
0	0.005	0.04	95

* Concentration in mm/3 ml of assay mixture

** Calculated. 3 ml of assay mixture contains 0.3 ml of enzyme.

FIGURE 14

Activity of Purified *M. sodonensis* Nuclease on
Polyadenylic Acid

Figure 14



0.06 ml of purified M. sodonensis nuclease was impregnated onto a filter paper disc, dried and placed on Diffusion Plate Assay Agar containing Poly A (0.5 mg/ml). Slides were incubated and developed as previously described.

FIGURE 15

Activity of Purified M. sodonensis Nuclease on
Several Polynucleotide Substrates

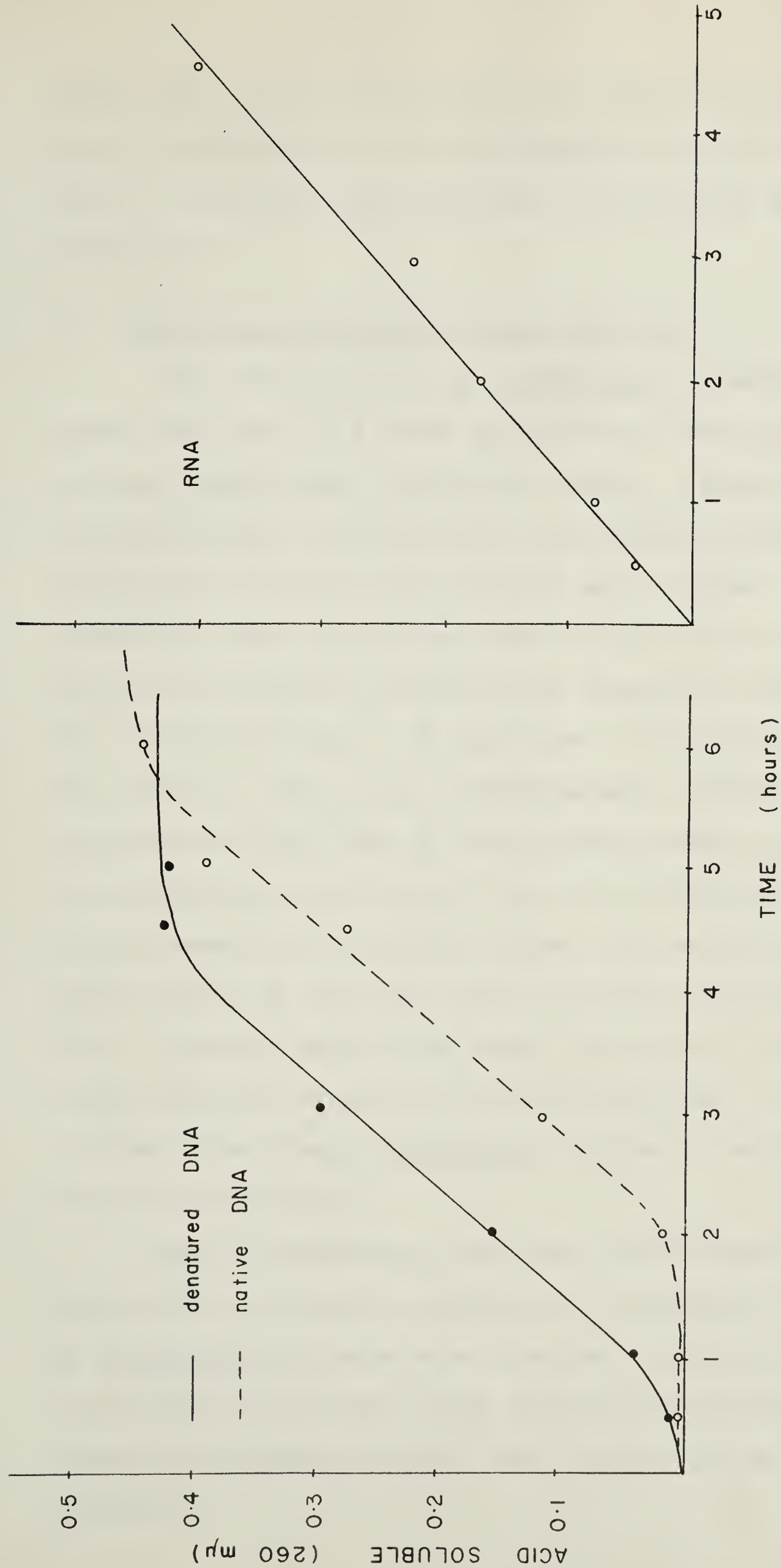


FIGURE 15

Table VIII, all of the 5' ribo and deoxyribo-nucleotides were attacked forming the corresponding nucleosides + P_i . The 3' ribonucleotides, pTp, ADP and ATP were not dephosphorylated.

5. Exonuclease and Endonuclease Activity

The activity of the M. sodonensis nuclease was compared with that of a known exonuclease (snake venom) and a known endonuclease (pancreatic DNase). Heat denatured A grade DNA was the substrate used and at the designated time intervals duplicate aliquots were removed, precipitated with cold 10% TCA and UTCA and the OD's at 260 m μ of the acid-soluble fraction were measured. Fig. 16 shows the results obtained. In the case of the pancreatic DNase the internal bonds of the molecules were attacked releasing oligonucleotides, some of which were soluble in 10% TCA (octanucleotides and below) but not in UTCA (which precipitates essentially anything larger than mononucleotides). Snake venom, on the other hand, attacks the molecule from the 3' hydroxyl end of the chain releasing 5' mononucleotides which are soluble in both TCA and UTCA. The results indicate that the M. sodonensis nuclease is exonuclease-like in its activity.

Table IX summarizes the data from an experiment in which standard reaction mixtures of pancreatic DNase and M. sodonensis nuclease were digested. At the designated time intervals aliquots were removed and analysed for change in viscosity and for UTCA soluble 260 m μ absorbing products.

TABLE VIII

Action of M. sodonensis Nuclease on Ribo
and Deoxyribomononucleotides

Substrate	MgP/ml digest	Substrate	MgP/ml digest
Cp	0	pC	.014
Ap	0	pA	.026
Gp	0	pG	.030
Up	0	pU	.017
Tp	-	pT	.020
dGp	-	dpG	.024
dAp	-	dpA	.031
dCp	-	dpC	.019
pTp	0	ADP	0
		ATP	0

FIGURE 16

Comparison of Activity of Various Nucleases
on Denatured DNA

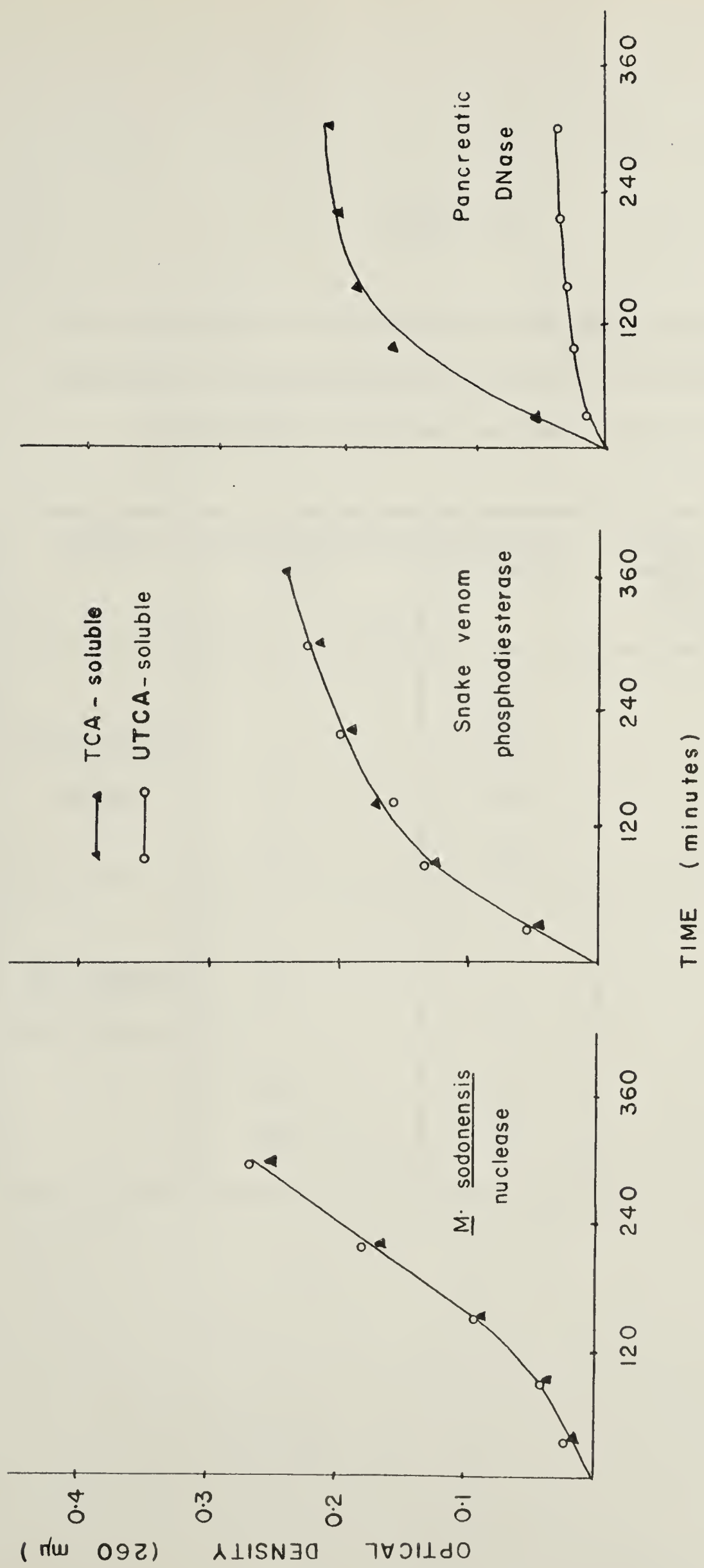


FIGURE 16

TABLE IX

A Comparison of Viscometric and Spectrophotometric Analyses of Depolymerase Activity of Purified M. sodonensis Nuclease and Pancreatic DNase

Enzyme	Reaction Time (min)	Relative Viscosity	Total OD Units Released into UTCA Soluble Fraction
Pancreatic DNase	0	3.17	0
	5	2.81	0
	10	2.48	0
	60	1.09	0
<u>M. sodonensis</u> Nuclease	0	3.3	0
	30	3.3	0
	60	2.67	.375
	120	2.27	.750
	180	1.64	1.50

The data are plotted in Fig. 17 and it may be seen that in the case of Pancreatic DNase (an endonuclease) there was a rapid decrease in viscosity preceding the appearance of UV absorbing, UTCA-soluble material. The M. sodonensis nuclease showed a more gradual decrease in viscosity concurrent with the appearance of UV absorbing UTCA-soluble products. The latter occurrence is typical of exonuclease activity and confirms the hypothesis that the M. sodonensis enzyme is an exonuclease.

6. Products of Reaction

The reaction products of M. sodonensis nuclease were analysed by paper chromatography and compared with those of snake venom phosphodiesterase (5' mononucleotides) and spleen phosphodiesterase (3' mononucleotides). The reaction mixtures were allowed to digest for 24 hr and 70 λ of the resultant digests were chromatographed on paper against mixtures of known nucleotides and nucleosides. The results can be seen in Fig. 18. Spleen phosphodiesterase released the 3' mononucleotides and snake venom the 5' mononucleotides while the products of M. sodonensis nuclease were the corresponding nucleosides with no detectable nucleotides. Analysis of the digests for inorganic phosphate showed that activity had resulted in a release of P_i by M. sodonensis nuclease whereas neither spleen nor snake venom phosphodiesterase exhibited this effect.

FIGURE 17

Comparison of Viscometric and Spectrophotometric
Analyses of Depolymerase Activity of Purified M.
sodonensis Nuclease and Pancreatic DNase

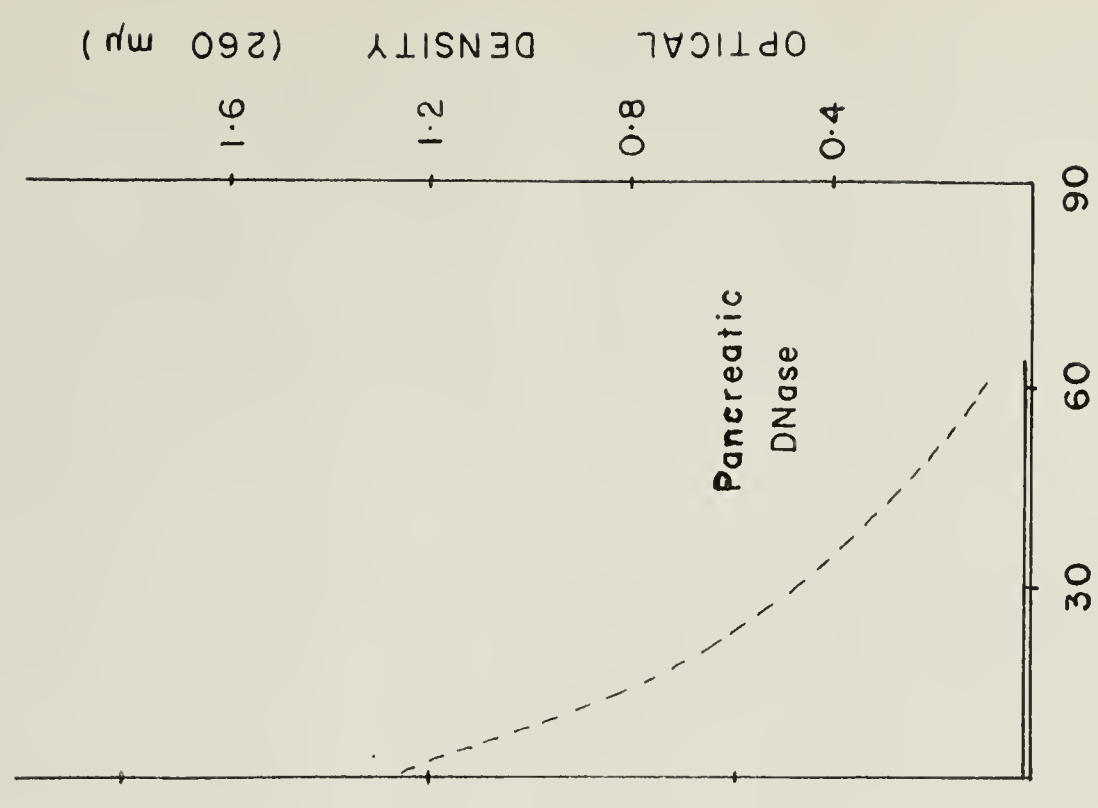
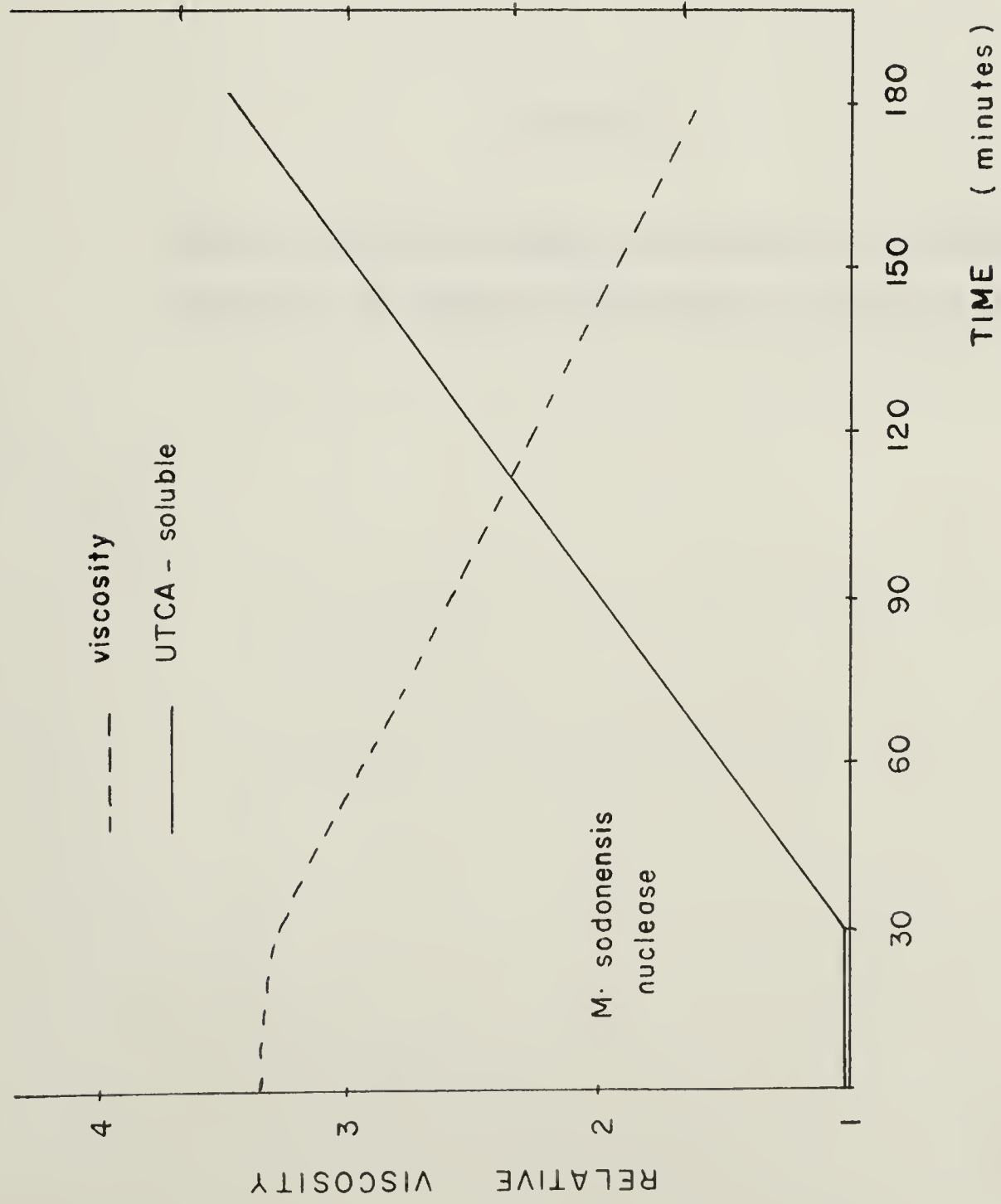
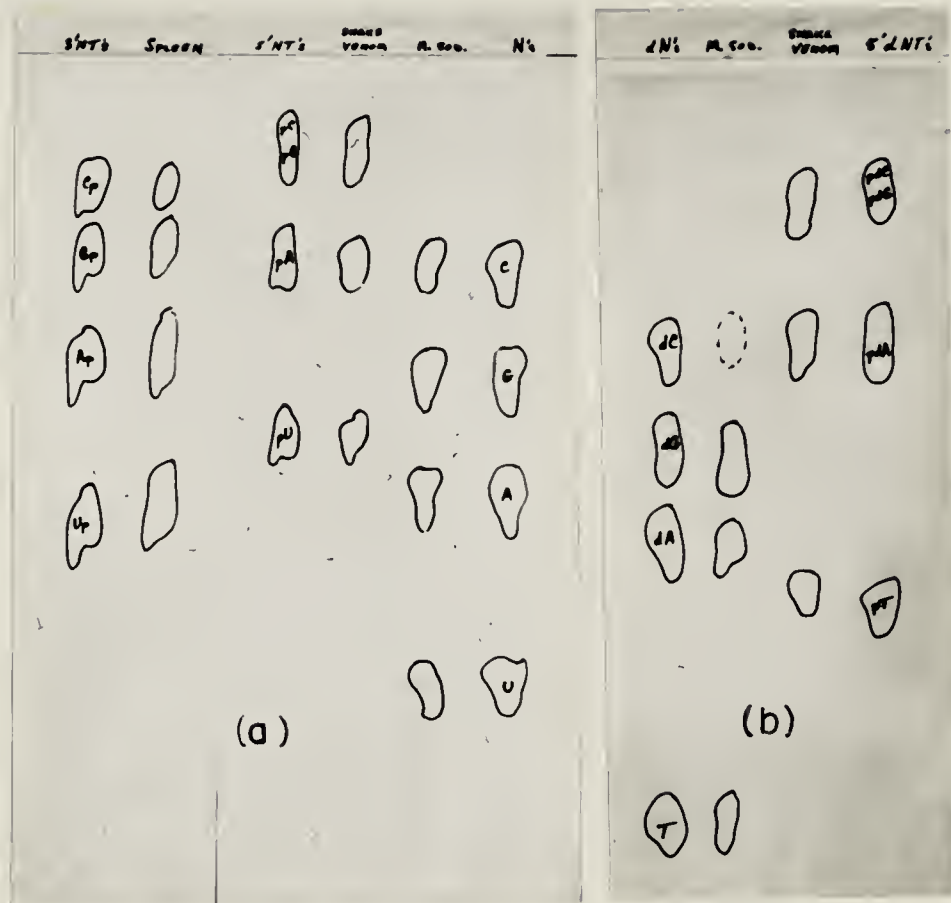


FIGURE 17

FIGURE 18

Paper Chromatographic Analysis of Reaction
Products of Several Enzymes on DNA and RNA

Figure 18



- (a) Products of digestion of RNA by M. sodonensis nuclease and spleen and snake venom phosphodiesterase.
- (b) Products of digestion of denatured DNA by M. sodonensis nuclease and snake venom phosphodiesterase.

7. Sedimentation Analysis

Fig. 19 is a photograph of the Schlieren diagram obtained upon ultracentrifugal analysis of a 0.1% solution of purified M. sodonensis nuclease in TRIS buffer. The upper photograph is a sedimentation velocity experiment (59,780 rpm) while the lower is the diagram obtained for an Archibald approach to sedimentation equilibrium (12,590 rpm).

The $S_{20,w}$ and molecular weight were calculated as follows:

(a) S

$$S \text{ measured} = 2.303 \times \frac{\frac{d \log x}{dt}}{60 \omega^2}$$

x = distance from angle of rotation

t = time in minutes

ω = angular velocity in radians/sec

Time (mins)	Dm*	Dc (Corrected distance cm) Dm - 2.138**	x (Corrected distance) Dc + 5.72***	$\log_{10} x$
0	0.7016	0.328	6.048	0.7816
16	0.7913	0.370	6.090	0.7846
32	0.8796	0.414	6.134	0.7878
48	0.9716	0.454	6.174	0.7906
64	1.0659	0.498	6.218	0.7937

* distance in cm from reference hole to peak

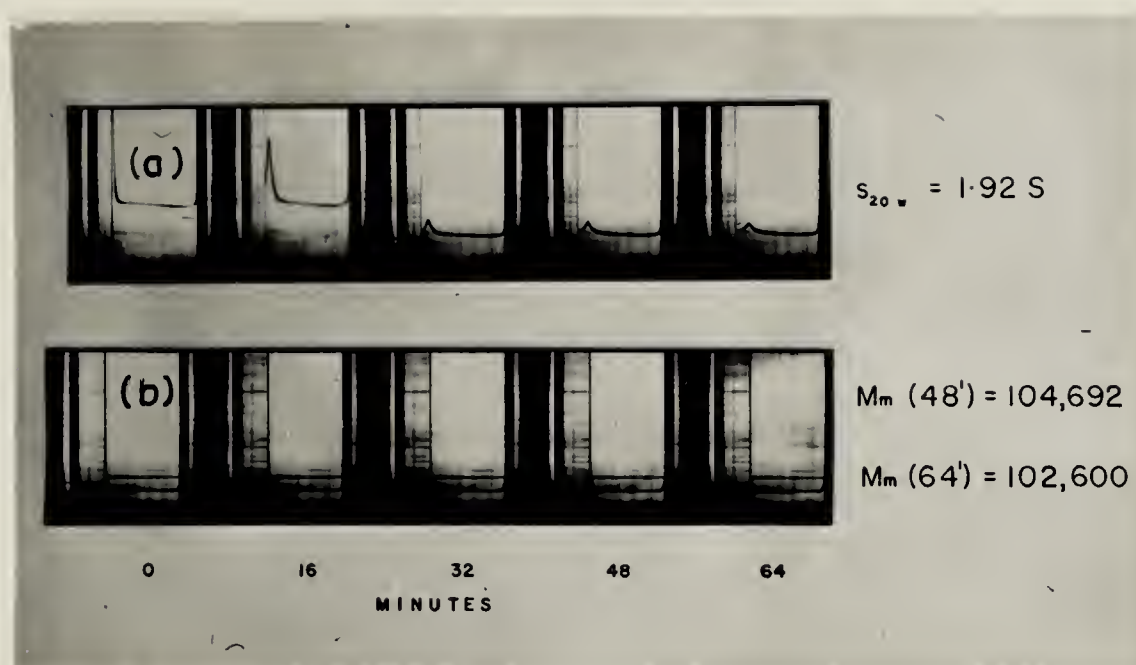
** magnification factor

*** distance from axis of rotation to reference hole

FIGURE 19

Schlieren Diagram of Sedimentation Analyses of
Purified M. sodonensis Nuclease

Figure 19



- (a) Sedimentation velocity
- (b) Archibald approach to sedimentation equilibrium

$$\frac{d \log_{10} x}{dt} = \frac{.7937 - .7816}{64} = 1.89 \times 10^{-4}$$

$$\omega^2 = \left(\frac{59,780}{60} \times 2\pi \right)^2 = 3.91 \times 10^7 \text{ radians/sec}$$

$$\therefore S_{\text{measured}} = \frac{2.303 \times 1.89 \times 10^{-4}}{60 \times 3.91 \times 10^7} = 1.85 \times 10^{-13}$$

$$(1S = 1 \times 10^{-13}) \quad \quad \quad = 1.85S$$

$$S_{20,w} = S_{\text{measured}} \left(\frac{\eta_t}{\eta_{20}} \right) \left(\frac{\eta}{\eta_o} \right) \left(\frac{1 - \bar{v} \rho_{20,w}}{1 - \bar{v} \rho_t} \right)$$

η_t = viscosity of H_2O at temperature t

η_{20} = viscosity of H_2O at temperature $20^\circ C$

$\frac{\eta}{\eta_o}$ = relative viscosity of solvent to H_2O

$\rho_{20,w}$ = density of H_2O at $20^\circ C$

ρ_t = density of solvent at temperature t

\bar{v} = partial specific volume = 0.73

$$\begin{aligned} S_{20,w} &= 1.85 \times \left(\frac{1.0259}{1.0050} \right) \left(\frac{1.0120}{1.0050} \right) \left(\frac{1 - 0.73 \times 0.9982}{1 - 0.73 \times 1.000} \right) \\ &= 1.92S \end{aligned}$$

(b) Molecular Weight

The peaks at 2 time intervals (48' and 64') were plotted and the information in the table obtained.

n	Rn/F	Xm (Rn/F + 5.70 cm)	Xm ² (cm ²)	48'		64'	
				Zn (cm)	Xm ² Zn (cm ³)	Zn (cm)	Xm ² Zn (cm ³)
0	0.47	6.17	38.068	1.30	49.488	1.20	45.681
1	0.48	6.18	38.192	1.20	45.830	1.15	43.920
2	0.49	6.19	38.316	1.08	41.381	1.05	40.231
3	0.50	6.20	38.440	0.89	34.211	0.90	34.596
4	0.51	6.21	38.564	0.65	25.066	0.72	27.766
5	0.52	6.22	38.688	0.48	18.570	0.58	22.439
6	0.53	6.23	38.813	0.25	9.703	0.41	15.912
7	0.54	6.24	38.937	0.18	7.008	0.28	10.902
8	0.55	6.25	39.062	0.05	1.953	0.19	7.421
9	0.56	6.26	39.187	0.01	0.391	0.10	3.918
10	0.57	6.27	39.312	--	--	0.02	0.786

$$\sum = 233.601$$

$$\sum = 253.572$$

n = interval

R_n = distance from reference point to interval

F = magnification factor

5.70 = distance from axis of rotation to reference point

Z_n = vertical height at interval in question

$$C_m = C_o - \frac{1}{X_m^2} \times \frac{dx}{F} \times \sum x_m^2 Z_n$$

C_m = concentration at meniscus

C_o = initial concentration, obtained by enlarging the peak from the sedimentation velocity run and calculating the area.

$$\frac{48 \text{ min}}{C_m} = 0.165 - \frac{1}{38.068} \times \frac{0.1}{10} \times 233.601 = 0.104$$

$$MW = \frac{RT}{(1-\bar{v}\rho)\omega^2} \times \frac{Zn}{C_m \cdot X_m}$$

$$T = 292^\circ \text{ K}$$

$$R = 8.314 \times 10^7 \text{ ergs/degree/mole}$$

$$\omega^2 = \left(\frac{12,590 \times 2\pi}{60} \right)^2 = 1.737 \times 10^6 \text{ radians/sec}$$

$$\rho \text{ of } 0.1 \text{ M HCl} = 1.000$$

$$MW = \frac{8.314 \times 10^7 \times 292}{(1-0.73 \times 1.000) \times 1.737 \times 10^6} \times \frac{1.3}{0.104 \times 6.17}$$

$$= 5.17 \times 10^4 \times \frac{1.3}{0.104 \times 6.17} = 104,692$$

64 min

$$C_m = 0.165 - \frac{1}{38.068} \times \frac{0.1}{10} \times 253.572$$

$$= 0.098$$

$$Mw = 5.17 \times 10^4 \times \frac{1.2}{0.098 \times 6.17} = 102,600$$

The sharpening of the peak in the sedimentation velocity experiment suggests either a system which is very concentration dependent, or the presence of a contaminant. This second possibility is the more likely since on further magnification a distortion of the leading edge of the peak was noted representing a maximum of 5% of the total.

The decrease of the molecular weight with time is another indication of contamination and is what would be expected if a heavier contaminant was separating out. Since the decrease in molecular weight is very small the molecular weight of the contaminant must be very close to that of the enzyme.

DISCUSSION

DISCUSSION

The results of this study have shown that M. sodonensis produces an extracellular enzyme which is capable of degrading polynucleotides and 5' mononucleotides. The release of the enzyme is clearly not due to autolysis since the maximum amount of enzyme per unit cell is obtained during the logarithmic phase of growth. As the cells enter the stationary phase the amount of enzyme per cell decreases, then is maintained at a constant level as the cells age. If autolysis was involved one would expect an increase of activity as the intracellular enzymes were released, but as this did not occur autolysis cannot be a major factor. A similar situation was reported by Hignett (1965) who found that the nuclease production by S. aureus was parallel to the growth curve and declined rapidly with the onset of the stationary phase.

Campbell et al (1961a) while working with M. sodonensis, found that this organism had a specific requirement for NH_4^+ in the growth medium. If it was not supplied exogenously it was made available by the deamination of glutamic acid \rightarrow α -ketoglutaric acid + NH_3 . These workers postulated that NH_4^+ was involved in pyrimidine synthesis via carbamyl phosphate and eventually into nucleic acids, and further evidence to support this has been obtained by Campbell (personal communication) with the use of $\text{N}^{15}\text{H}_4\text{Cl}$. This study has shown that NH_4^+ is also stimulatory to the production of nuclease, and the addition of NH_4^+ to the synthetic medium results in a yield of enzyme per unit cell

in excess of that produced in an undefined medium such as TCS broth. The appearance of small amounts of enzyme after 36 hours in the NH_4^+ deficient synthetic medium is probably due to the deamination of glutamic acid, and as NH_4^+ becomes available both enzyme production and growth are increased. Since enzyme production is at a maximum during periods of rapid nucleic acid synthesis its function probably lies in nucleic acid turnover. However the stimulation of enzyme production cannot be explained simply by an intracellular accumulation of DNA. Campbell, Evans, Perry and Niven (1961b) demonstrated a two-fold increase in intracellular DNA when adenine or adenosine was incorporated into the culture medium. It has since been demonstrated in this laboratory (unpublished data) that the substitution of adenine for NH_4^+ in the synthetic medium results in a depression of enzyme production and therefore increased concentration of intracellular DNA is not the inducing factor involved. It is suggested that, since adenosine is an end product of the enzyme activity, its presence in excess may result in end product inhibition or repression of enzyme production. NH_4^+ has been shown to be utilized in the de novo synthesis of the purine and pyrimidine nucleotides (e.g. pG and pU) in this organism and, since the enzyme possesses monoesterase activity, these nucleotides are substrates and possibly the inducers of enzyme production.

The reasons for the extracellular release of the enzyme are not known but it cannot be functioning merely as a scavenger for extracellular polynucleotides since it is

released into media in which no substrate is present upon which it can act. The presence of the enzyme, however, in media containing polynucleotides or nucleotides renders these materials, which normally could not pass the cell permeability barrier, readily available to the cell. Since the enzyme could degrade any transforming DNA which might be present, another possible role might be in maintaining the genetic immunity of the cell.

Although the yield per unit cell in TCS broth was lower than that in synthetic medium +NH₄⁺, the total amount produced was considerably higher and this then was the medium of choice for enzyme recovery. A recovery of 42.6% of the total initial activity was obtained after purification procedures. The figures given were those obtained under the best conditions but recovery varied from 28% to 42.6% with different experiments. A 90-100% recovery after dialysis and gel filtration was the general rule but the recovery from DEAE-cellulose ranged from 65-100% in a number of experiments. A recovery of 42.6% and a 1400 fold purification compares very favorably with that obtained with other microbial nucleases. Eaves et al (1963) recovered 44.9% of the initial activity of Serratia marcescens nuclease but were able to obtain only a 233 fold purification. Micrococcal nuclease was purified 460 fold but only 5% of the initial activity was recovered (Alexander et al, 1961). Fourteen % of the phosphatase-exonuclease activity of E. coli was recovered by Richardson and Kornberg (1964) who obtained a 1300 fold purification of the enzyme.

The single peak obtained after gel filtration and the single band of activity after electrophoresis, as well as the increase in specific activity are all indications of a fairly high degree of purification. There is probably a small amount of contaminant present as evidenced by the sedimentation analyses. The sharpness of the peak, the distortion of the leading edge of the peak and the decrease in molecular weight with time are all indications of a contaminant but it constitutes an estimated maximum of 5% of the total material and may, in fact, prove to be inactivated enzyme. The contaminant cannot be separated on the basis of charge since both ion exchange chromatography and electrophoresis yielded a single peak. It must be very close to the enzyme in molecular weight since a single peak was obtained with gel filtration and the decrease with time of the calculated molecular weight in the Archibald run was very small.

The initial finding of 2 peaks of activity on DEAE-cellulose was shown to be an artifact due to the high concentration of salt in the undialyzed material. After dialysis and rechromatography of Fraction I there was a shift of activity to those fractions which were eluted at 0.15 - 0.25 M NaCl. Additional evidence may be drawn from Table V. During the purification no activity is lost upon dialysis and the activity of the dialysed fraction can be completely recovered from DEAE-cellulose as a single peak at a concentration of 0.15 - 0.25 M NaCl.

Studies on the depolymerase activity of the purified

enzyme revealed that, in contrast to the Micrococcal nuclease from S. aureus, it is heat sensitive and is inactivated after 5 minutes at 55°C. One of the distinguishing characteristics of Micrococcal nuclease is that it is quite stable to heat, and 100% of the activity of crude culture supernatant is retained after heating at 95°C for 15 minutes (Cunningham et al, 1956). Alexander et al (1961) found that the $(\text{NH}_4)_2\text{SO}_4$ fraction was stable for 10 minutes at 89°C, while the purified material still retained 30% of its activity after 5 minutes at 100°C. The pH optimum of M. sodonensis nuclease is similar to that of Micrococcal nuclease, ranging from 8.2 to 9.0 with maximum activity at about 8.6 (Cunningham et al, 1956; Alexander et al, 1961).

The cation requirement of the M. sodonensis depolymerase is unique in that both Mg^{++} and Mn^{++} are essential and neither will function alone to support activity. Neither Mg^{++} nor Mn^{++} can be replaced by Ca^{++} but the addition of Ca^{++} in the presence of the other cations has a stimulatory effect. The concentration of Mn^{++} is critical and any increase above the optimum level becomes inhibitory. Micrococcal nuclease requires only Ca^{++} for activation (Cunningham et al, 1956; Alexander et al, 1961) and this requirement cannot be replaced by Mg^{++} . The 4 nucleases of E. coli, on the other hand, are all Mg^{++} requiring and, with the exception of Exonuclease I, the Mg^{++} can be partially replaced by Mn^{++} (Lehman et al, 1960, 1962, 1963).

In addition to their role in the activation of M. sodonensis nuclease, Mg^{++} and Ca^{++} also have a stabilizing

effect. When dialysis of the enzyme was carried out against distilled H₂O approximately 50% of the activity was recovered after chromatography on DEAE-cellulose while dialysis against 0.01 M TRIS buffer resulted in a 68% recovery. Activity was 100% recoverable, however, after dialysis against 0.01 M TRIS buffer which was 0.004 M with respect to Mg⁺⁺ and Ca⁺⁺. This seems to indicate that these cations help to maintain a stable active configuration of the protein.

The depolymerase activity of M. sodonensis is non-specific with DNA, RNA and Poly A serving as substrates. The rate of attack on RNA was slightly slower than that on DNA, but there was no significant difference, and, while a precise quantitative estimation of activity was not carried out on Poly A it appears to be as good a substrate as the other polynucleotides. The long lag phase with native DNA can be shortened by pre-incubation of the substrate mixture at 37°C. This preincubation apparently does not cause denaturation, since no hyperchromicity is noted, but may be merely a further solubilization of the highly polymerized substrate which is difficult to get into solution. The lag period obtained with DNA, which does not occur with RNA, may indicate the necessity of an initial unwinding of the strands. The rates of attack on native and denatured DNA, after the lag period, are identical. This is in contrast to the situation with Micrococcal and E. coli nucleases which have been found to preferentially attack one or the other form of the DNA substrate. Micrococcal nuclease attacks denatured DNA at a

faster rate than native (Dirksen et al, 1960; von Heppel and Felsenfeld, 1964) as does E. coli Exonuclease I (Lehman and Nussbaum, 1964), while Exonuclease II and III have a distinct preference for native DNA (Richardson et al, 1964). The nucleases of E. coli are specific for DNA but Micrococcal nuclease will attack RNA as well.

The mode of attack of M. sodonensis nuclease on DNA and RNA is that of an exonuclease, as is evidenced by the comparative studies carried out with known exo- and endonucleases. From UTCA - TCA data it is obvious that the products released by the enzyme are not larger than mononucleotides since they are soluble in both TCA and UTCA as are those products released by snake venom phosphodiesterase. Quite a different result is noted with pancreatic DNase (an endonuclease) which releases larger fragments which are soluble in TCA but not in UTCA. Confirmation of the exonuclease activity is obtained from the viscosity data which shows a gradual decrease in viscosity concurrent with a release of UTCA-soluble, 260 mμ absorbing products. This is in contrast to the findings with pancreatic DNase where a rapid decrease in viscosity occurs before any appearance of UTCA-soluble products. Lehman and Richardson (1964) used the same approach as evidence for the exonucleolytic activity of E. coli Exonuclease II when they compared viscosity with the appearance of acid-soluble P³².

Analysis of the reaction products of M. sodonensis nuclease revealed the presence of nucleosides + P_i. The

appearance of P_i is due to the associated phosphomonoesterase activity which is specific for the 5' nucleotides. This specificity suggests that the enzyme attacks the polynucleotide from the 3' hydroxyl end of the chain releasing the 5' mononucleotides which are then dephosphorylated. The enzyme appears to have no base preference since the rate of attack remains constant throughout the reaction and all 4 nucleosides are found in the reaction products. Although further studies are necessary using substrates of known composition and known end groups, some assumptions can be made on the specificity of the depolymerase activity. The addition of an ionizable group to the 5' phosphate of adenylic acid (e.g. ADP, ATP) inhibits the monoesterase activity and indicates the specificity of the enzyme for the pN.pN linkage. The inability of the monoesterase to attack pTp is indicative of the inhibitory effect of the 3' phosphoryl group and the possibility exists that this inhibition may extend to the depolymerase activity. It is suggested that (as with snake venom phosphodiesterase, Razzell et al, 1959) in order for the nuclease to attack the polymer a free 3' hydroxyl must be present since no pNp was ever detected in the reaction products.

Table X summarizes and compares some of the properties of E. coli and Micrococcal nucleases with those of the M. sodonensis enzyme.

The calculated molecular weight of the M. sodonensis nuclease is reasonable in view of its behavior on Sephadex G-100. The active fraction appeared immediately after the void volume which indicates a molecular weight of greater

TABLE X

A Comparison of Depolymerase Activity of Several Microbial Nucleases*

Source	<u>M. sodonensis</u>		<u>E. coli</u> Endonuclease I		<u>E. coli</u> Exonucleases		
	Extracellular	Micrococcal	Intracellular	Intracellular	I	II	III
pH optimum	8.6	8.6	8.0	9.5	9.2	7.0	
Cation requirement	Mg ⁺⁺ 1.3x10 ⁻² M Mn ⁺⁺ 1.7x10 ⁻³ M Ca ⁺⁺ 1.7x10 ⁻³ M	Ca ⁺⁺ 1x10 ⁻² M	Mg ⁺⁺ 7x10 ⁻³ M (Mn ⁺⁺)	Mg ⁺⁺ 1.5x10 ⁻³ M	Mg ⁺⁺ 7x10 ⁻³ M (Mn ⁺⁺)	Mg ⁺⁺ 1x10 ⁻² M (Mn ⁺⁺)	
Heat Stability	100% inactivation, 5 min at 55° C	70% inactivation, 5 min at 100° C	80% inactivation, 10 min at 100° C	-	-	90% inactivation, 15 min at 37° C	
Substrate	RNA, native DNA, denatured DNA	RNA, preference for denatured DNA	Native DNA	Denatured DNA	Preference for native DNA	Native DNA	
Type of Attack	Stepwise from 3' hydroxyl end of chain	Endonucleolytic	Endonucleolytic	Stepwise from 3' hydroxyl end of chain	Stepwise from 3' hydroxyl end of chain	Stepwise from 3' hydroxyl end of chain	
Products	Nucleosides + P _i	Mono, di and oligonucleotides terminating in 3' phosphate	Oligonucleotides (average chain length of 7) terminating in 5' phosphate	Mono & dinucleotides terminating in 5' phosphate	5' mononucleotides	P _i , 5' mononucleotides and large molecular weight, single stranded oligonucleotides	
Required end group on DNA	--- P ↓ P ↓ OH	-	-	--- P ↓ P ↓ OH	--- P ↓ P ↓ OH	--- P ↓ P ↓ OH or --- P ↓ P ↓ P	
Phosphomonoesterase activity	Active on 5' mononucleotides	-	-	-	-	Initial attack removes P _i from 3' phosphoryl terminus	

* Data was obtained from Lehman et al (1960, 1962, 1964), Richardson et al (1964), Cunningham et al (1956) and Alexander et al (1961).

than 100,000. The heat sensitivity of the enzyme is also evidence for a larger molecule. Small molecules have a tendency to be more heat stable (Dixon and Webb, 1964) as is noted with Micrococcal nuclease with a molecular weight of 12,000 and RNase with a molecular weight of 13,700. There is a discrepancy in the M. sodonensis data between the $S_{20,w}$ and the molecular weight, the value of 1.92S being abnormally low for a molecular weight of around 100,000. Micrococcal nuclease has an $S_{20,w}$ of 1.8 and a molecular weight of 12,000 (Alexander et al, 1961) close to that of RNase with a molecular weight of 13,700 and an $S_{20,w}$ of 1.88 (Schachman, 1957). Myoglobin has a molecular weight of 16,900 and an $S_{20,w}$ of 2.0S while a protein such as canavalin with a molecular weight of 113,000 has an $S_{20,w}$ of 6.4 (Alexander and Johnson, 1949). The low $S_{20,w}$ value for the M. sodonensis nuclease may be a result of a marked assymetry of the molecule but more studies are necessary to resolve this.

Phosphomonoesterase activity was found to be associated with the depolymerase at all stages of the purification procedure and was inseparable from it by any of the methods used. Although the possibility of 2 separate enzymes does exist there is good evidence for the existence of one enzyme with 2 activities. The fact that the 2 activities were purified to the same extent argues for the existence of one enzyme as does the inability to separate the 2 activities either electrophoretically or by gel filtration. Individual fractions of the active peak

from Sephadex G-100 were analysed for monoesterase and depolymerase activity and the ratio of the 2 activities remained constant in each fraction tested. The first fraction to exhibit depolymerase activity also contained monoesterase activity and there was no accumulation of either activity at the leading or trailing edge of the peak.

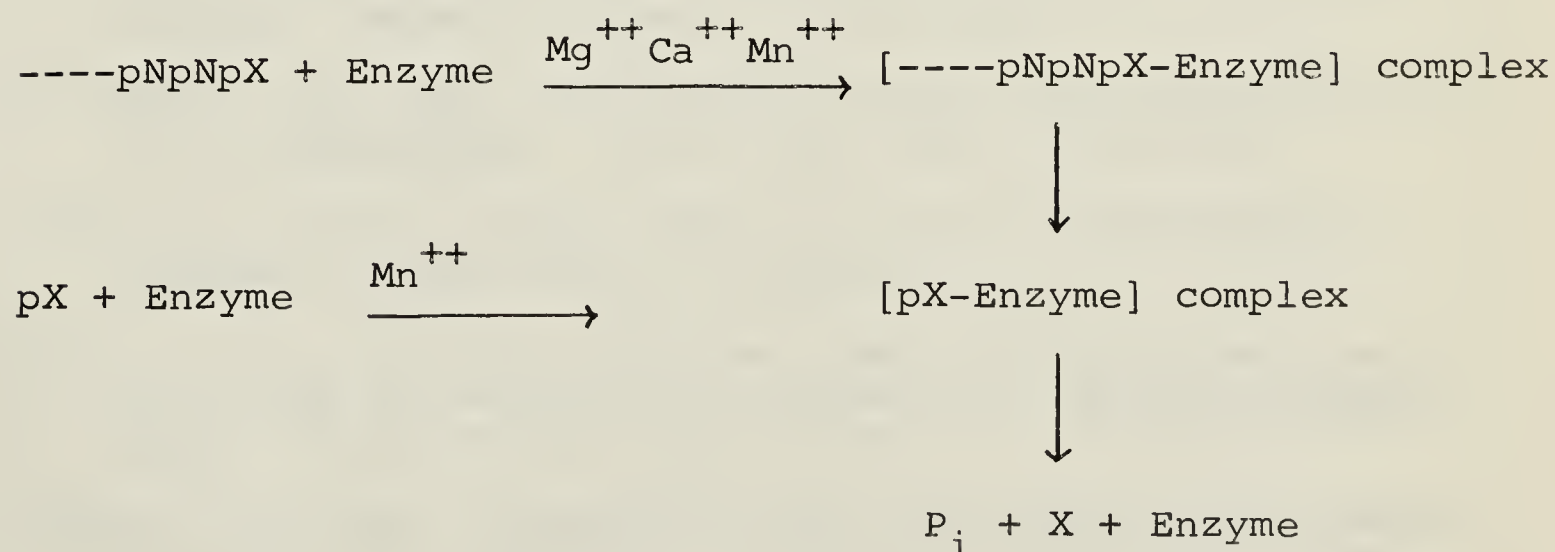
Exonuclease III of E. coli (Richardson et al, 1964) has an associated phosphatase activity but it differs from M. sodonensis in that it removes the 3' phosphate from the intact polymer, thus enabling the exonuclease activity to proceed with the release of 5' mononucleotides. This has been shown to be one enzyme with 2 activities and Richardson and his coworkers gave as evidence for a single enzyme the identical pH requirement, cation requirement and heat inactivation curves of the 2 activities.

The 2 activities of M. sodonensis exhibit the same pH dependency but there are differences in the heat inactivation curves and the cation requirements. These differences could indicate 2 different enzymes but do not necessarily discount the possibility of 1 enzyme with 2 activities. It is suggested that, in view of the data, a single protein exists with 2 binding sites. One of these sites is involved in the complexing of the enzyme with both the polynucleotide and the mononucleotide substrate as is indicated by the identical requirement for and the toxic effect of Mn^{++} , as well as the identical pH optima. The second binding site is necessary only for the complexing of the enzyme to the polynucleotide sub-

strate, is Mg^{++} and Ca^{++} requiring and is more susceptible to configurational changes when the protein is heated.

From the data obtained it is suggested that M. sodon-
ensis nuclease is a single enzyme with both depolymerase
and phosphomonoesterase activity. Its mode of attack is
that of an exonuclease and its attack on the polynucleotide
polymer is postulated as proceeding from the 3' hydroxyl
end of the chain with the release of nucleosides + P_i .

The identical Mn^{++} requirements for both depolymer-
ase and monoesterase activity, the identical pH dependency,
plus the fact that at no time are free nucleotides detect-
able among the reaction products leads to the postulation
of a single protein with at least one common binding site
for the different substrates. The mechanism of action
shown in the following diagram is suggested rather than a
system wherein the 5' nucleotide is released and recom-
plexed with another enzyme, or another site on the same
enzyme, and dephosphorylated.



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